

DNA Procedures for Reporting Serological Testing Results

1 Scope

These procedures apply to DNA personnel who prepare *Laboratory Reports* (7-1, 7-1 LIMS, 7-273, or 7-273 LIMS) containing serological testing results.

2 Reporting Guidelines

As a part of its quality program, the DNA Units have developed standard operating procedures (SOPs) for the various testing methods used in the serological examination of evidence in the DNA Casework Unit (DCU) and the Biometrics Analysis Unit (BAU). The combination of the results from different procedures must be taken into account for the examiner to develop a conclusion.

To report the results of serology examinations, the examiner must consider all tests conducted. Combinations of negative, positive, and inconclusive test results not described below may be encountered, or one of the tests may not have been conducted (e.g., for the purposes of sample conservation). In such cases, the statements below should be used for guidance as to how to report the specific combination of results encountered.

Requirements for the formatting and administrative content of *Laboratory Reports* are contained in the FBI Laboratory Practices for Preparing, Reviewing, and Issuing Laboratory Reports and Retaining Records in Forensic Advantage and the DNA Procedures for Case File Assembly and Reviews. Results of serology and DNA testing may be combined into a single *Laboratory Report*.

2.1 Reporting Blood Examination Results

The determination of whether blood is present on an item is dependent on the results of a visual examination, the phenolphthalin test, and/or the Takayama test. The report should generally use the following language based on the combination of these test results.¹

2.1.1 Negative Blood Results

2.1.1.1 When the item is visually examined and no areas of serological value were observed and/or further tested, the following statement should generally be included in the report:

A visual examination was conducted on item 1; however, no areas of serological value were observed.

¹ For a given item of evidence, only the results for the stain on that specimen that yielded the most information with respect to the blood examinations conducted are reported.

It is noted that if any other blood examination(s) are conducted, the results of the visual examination are not reported.

2.1.1.2 When the phenolphthalin test is negative for an item and the Takayama test is not conducted, the following statement should generally be included in the report:

*Blood was not detected on item 2.**

with the following explanatory endnote:

** This conclusion is based on a negative presumptive test. Insufficient quality and/or quantity of biological material may affect the ability to detect blood.*

2.1.2 Presumptively Positive Blood Results

When the phenolphthalin test is positive for an item and the Takayama test is either not conducted or negative, the following statement should generally be included in the report:

*Blood was indicated on item 3.**

with one of the following explanatory endnotes, as appropriate:

**This conclusion is based on a positive presumptive test. Further confirmatory testing was not conducted. This result provides an indication that blood may be present on an item, but does not constitute an identification of blood. Insufficient quality and/or quantity of biological material may affect the ability to detect blood.*

**This conclusion is based on a positive presumptive test and a negative confirmatory test. These results provide an indication that blood may be present on an item, but do not constitute an identification of blood. Insufficient quality and/or quantity of biological material may affect the ability to detect blood.*

2.1.3 Confirmatory Blood Results

When the Takayama test is positive for an item, regardless of the results of the phenolphthalin test, the following statement should generally be included in the report:

*Blood was identified on item 4.**

with one of the following explanatory endnotes, as appropriate:

**This conclusion is based on positive results for both the presumptive and confirmatory tests.*

**This conclusion is based on a positive result for the confirmatory test. The presumptive test was inconclusive, which may occur when an unknown substance(s)*

prevented the interpretation of the presumptive test.

2.1.4 Inconclusive Blood Results

When the phenolphthalin test is inconclusive for an item and the Takayama test is either negative or not conducted, the following statement should generally be included in the report:

No determination can be made regarding the presence or absence of blood on item 5..*

with one of the following explanatory endnotes, as appropriate:

**This conclusion is based on an inconclusive presumptive test, which may occur when an unknown substance(s) prevented the interpretation of the presumptive test.*

**This conclusion is based on an inconclusive presumptive test, which may occur when an unknown substance(s) prevented the interpretation of the presumptive test. The confirmatory test was negative.*

2.2 Reporting Semen Examination Results

The determination of whether semen is present on an item is dependent on the results of a visual examination with an alternate light source, the acid phosphatase (AP) test, and/or the microscopic search for a sperm cell. The report should generally use the following language based on the combination of these test results.²

2.2.1 Negative Semen Results

2.2.1.1 When the item is visually examined with an alternate light source and no areas of serological value were observed and/or further tested, the following statement should generally be included in the report:

A visual examination was conducted on item 6; however, no areas of serological value were observed.

It is noted that if any other semen examination(s) are conducted, the results of the visual examination are not reported.

2.2.1.2 No semen is detected on an item if the results of every test conducted are negative. The following statement should generally be included in the report:

*Semen was not detected on item 7.**

² For a given item of evidence, only the results for the stain on that specimen that yielded the most information with respect to the semen examinations conducted are reported.

with one of the following explanatory endnotes, as appropriate:

**This conclusion is based on a negative presumptive test. Insufficient quality and/or quantity of biological material may affect the ability to detect semen.*

**This conclusion is based on a negative microscopic examination for a sperm cell. Microscopic examinations may not detect semen from azoospermic (e.g., vasectomized) males. Insufficient quality and/or quantity of biological material may affect the ability to detect semen.*

**This conclusion is based on a negative presumptive test and a negative microscopic examination for a sperm cell. Microscopic examinations may not detect semen from azoospermic (e.g., vasectomized) males. Insufficient quality and/or quantity of biological material may affect the ability to detect semen.*

2.2.2 Presumptively Positive Semen Results

When the AP test is positive and a sperm search is either not conducted or is negative, the following statement should generally be included in the report:

*Semen was indicated on item 8.**

with one of the following explanatory endnote, as appropriate:

**This conclusion is based on a positive presumptive test result. Further confirmatory testing was not conducted. This result provides an indication that semen may be present on an item, but does not constitute an identification of semen. Insufficient quality and/or quantity of biological material may affect the ability to detect semen.*

*“*This conclusion is based on a positive presumptive test result and a negative microscopic examination for a sperm cell. These results provide an indication that semen may be present on an item, but do not constitute an identification of semen. Microscopic examinations may not detect semen from azoospermic (e.g., vasectomized) males. Insufficient quality and/or quantity of biological material may affect the ability to detect semen.”*

2.2.3 Confirmatory Semen Results

Semen is confirmed on an item when the microscopic sperm search is positive, regardless of the results of the other examinations. The following statement should generally be included in the report:

*Semen was identified on item 9.**

with one of the following explanatory endnotes, as appropriate:

**This conclusion is based on the microscopic observation of a sperm cell.*

**This conclusion is based on a positive presumptive test and the microscopic observation of a sperm cell.*

**This conclusion is based on a negative presumptive test and the microscopic observation of a sperm cell.*

2.2.4 Inconclusive Semen Results

If the AP result for an item is inconclusive, and a sperm search is either not conducted or is negative, the following statement should generally be included in the report:

*No determination can be made regarding the presence or absence of semen on item 10.**

with one of the following explanatory endnotes, as appropriate based on the tests conducted:

**This conclusion is based on an inconclusive presumptive test, which may occur when an unknown substance(s) prevented the interpretation of the presumptive test.*

**This conclusion is based on an inconclusive presumptive test, which may occur when an unknown substance(s) prevented the interpretation of the presumptive test. The confirmatory test was negative.*

2.2.5 Slide Created without Microscopic Examination

For evidence where samples are sent directly to DNA testing (e.g., Sexual Assault Kit swabs), a slide may be made during the differential extraction process but may not be serologically examined. When a male CODIS eligible DNA profile is developed from an item of evidence for which a slide was created, the following statement should be included in the *Laboratory Report* remarks to inform the contributor that serology testing may be requested if needed in the future. When a male profile is not developed, the statement does not need to be added.

As part of the analytical process, a slide was prepared from item 11 for possible semen identification. The slide was not examined at this time; however, it can be resubmitted if future examinations are needed.

3 References

United States. Department of Justice. Office of Legal Policy. Forensic Science. *Department of Justice Uniform Language for Testimony and Reports for the Forensic Serological Examinations*. Retrieved from the Department of Justice Web site:
<https://www.justice.gov/olp/uniform-language-testimony-and-reports>.

Rev. #	Issue Date	History
5	02/28/18	Added scope. Incorporated BAU into introduction. Added guidance on reporting when a slide was created but not tested.
6	03/27/19	Updated reporting language to incorporate changes based on the DOJ Serology ULTR requirements.

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 03/25/2019

Acting BAU Chief

Date: 03/25/2019

DCU Chief

Date: 03/25/2019

QA Approval

Quality Manager

Date: 03/25/2019

DNA Procedures for the Presumptive Testing of Blood

1 Scope

These procedures apply to DNA personnel that screen evidentiary items in the DNA Casework Unit (DCU) for the possible presence of blood.

2 Equipment/Materials/Reagents

- Swabs, Puritan or equivalent
- Water, Reagent Grade or equivalent
- Known Positive (KP) blood control, dilute blood
- Phenolphthalin solution
- Hydrogen peroxide (H₂O₂), 3% solution

Refer to the appropriate DNA QA procedure for reagent and control preparation information.

3 Standards and Controls

The phenolphthalin solution and hydrogen peroxide must be tested prior to first daily use on evidentiary items to verify the continued detection efficacy. A known positive (KP) and known negative (KN) must be tested by the biologist, each day, prior to using the phenolphthalin test reagents for casework.

A KP blood control is a sample of dried human blood. A KP prepared with diluted blood (generally diluted to 1:250 with water) will be tested following the procedures in this document. The diluted KP is used to reestablish the necessary swabbing pressure and the expected test result for less concentrated stains. Internal studies indicate that blood diluted to 1:250 was consistently detected using these procedures. A neat blood sample may be tested to demonstrate the expected color changes that occur with a concentrated blood stain but is not required to be tested each day. A clean swab will be tested as the KN blood control. Aliquot(s) of the phenolphthalin reagents that do not yield a positive reaction (i.e., a distinct pink color change) with a KP blood control or that yield a positive reaction with a KN blood control must not be used for casework.

4 Sampling

Items with an indication that blood may be present (e.g., red-brown staining, scenario information), may be tested using the phenolphthalin test. Any area of potential staining will be spot tested and, at minimum, areas that test positive will be described in the case notes.

5 Procedures

Refer to DNA Procedures Introduction (i.e., DNA QA 600) for applicable laboratory quality assurance and cleaning instructions.

Ensure the appropriate fields (i.e., reagents, KP) in STACS are completed from any network computer, as necessary.

5.1	Using a new, clean swab moistened to dampness with reagent grade water, rub the stained area until a visible amount of stain has been transferred to the swab, or the swab appears matted.	
5.2	Add ~1-3 drops of phenolphthalin solution to the swab.	
5.3	Observe the swab tip for any color change for ~3 seconds.	
5.3.1	If no color change is observed, continue with the procedure.	
5.3.2	If a pink color is observed, the procedure should be stopped at this step. Record this result as inconclusive (INC). Consult an Examiner prior to conducting any additional testing.	
5.3.3	If an unexpected non-pink color is observed, the procedure should be stopped at this step. Record this result as INC. Consult an Examiner prior to conducting any additional testing.	
<p>Note: Heavily saturated blood stains are known to turn greenish gray after addition of the phenolphthalin solution.</p>		
5.4	Add ~1-3 drops of 3% hydrogen peroxide solution to the swab	
5.5	Observe the swab for a color change within 10 seconds. Record the test results as listed below: The observation of a pink color... Positive (POS) The observation of no color... Negative (NEG) The observation of a non-pink color... Inconclusive (INC)	

Generally, the color change will occur instantly. Due to oxidation, the swabs used for negative stains may turn pink after ~2 minutes. It is generally expected that most swabs inoculated with both phenolphthalin solution and 3% hydrogen peroxide solution may display a pink color within approximately 30 minutes of their exposure to air.

The presence of a non-pink color could mask any potential pink color that would result from the presence of blood. If such an observation is made, an Examiner should be consulted prior to conducting any additional testing.

The reporting language used for the results from this testing and others is contained within the appropriate procedure (i.e., Sero 100) in the *DNA Procedures Manual*.

6 Reagent Quality Control

6.1 Each new batch of phenolphthalin solution and 3% hydrogen peroxide solution will be tested for efficacy at the time of its preparation using the analytical procedure. The phenolphthalin solution and 3% hydrogen peroxide may be tested concurrently or independently with an in use lot of the counterpart reagent.

6.1.1 A positive test result (i.e., a pink color) for the KP blood control establishes that the new batch of phenolphthalin solution and/or 3% hydrogen peroxide solution is yielding the expected positive result. A new batch of phenolphthalin solution and/or 3% hydrogen peroxide solution that does not yield a positive reaction with a KP blood control must not be used for casework.

6.1.2 A negative test result (i.e., no color) for the KN blood control establishes that the new batch of phenolphthalin solution and/or 3% hydrogen peroxide solution is not itself yielding a positive result (i.e., a pink color) in the absence of blood. A new batch of phenolphthalin solution and/or 3% hydrogen peroxide solution that yields a positive reaction (i.e., a pink color) with a KN blood control must not be used for casework.

6.2 If the expected results for both the KP and KN blood controls are obtained using the new batch of phenolphthalin solution and/or 3% hydrogen peroxide solution, that preparation of phenolphthalin solution and/or 3% hydrogen peroxide solution may be used for casework.

7 Calculations

Not applicable.

8 Measurement Uncertainty

Not applicable.

9 Limitations

9.1 A positive reaction with the phenolphthalin test provides a presumptive indication that blood may be present on an item but does not constitute an identification of blood. A confirmatory testing procedure (i.e., Takayama) is required to identify the presence of blood in a questioned stain.

9.2 A positive phenolphthalin test is not required for the identification of blood. The utility of the phenolphthalin test is to determine the stain(s) that may be blood, so that further testing (e.g., confirmatory test, DNA testing) can be focused on those stains most likely to yield additional information.

9.3 For the phenolphthalin test to be considered positive, a pink color must be observed within 10 seconds of the addition of the 3% hydrogen peroxide solution. Due to the nature of this chemical reaction (i.e., reduction-oxidation), any pink color observed after this period may be mediated by other oxidizing agents (e.g., oxygen).

9.4 Should the color of an item preclude the interpretation of the phenolphthalin test, or should the test give an inconclusive result, a confirmatory testing procedure may be used to identify the presence of blood in a questioned stain.

9.5 While a negative phenolphthalin test indicates that no blood was detected in a stain, the failure to detect blood is not the basis for an absolute determination that blood was not present. Negative test results may be obtained in the presence of blood when it is present in a quantity below the detection limit of the phenolphthalin test.

9.6 Presumptive blood testing should not be conducted on areas of items with potential value for latent print examination.

10 Safety

10.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. All DNA personnel who work with such material will follow the “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP)” found in the most current version of the *FBI Laboratory Safety Manual*.

10.2 Refer to the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual* for important personal safety information prior to conducting this procedure.

10.3 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in this procedure as well as the biohazardous wastes generated.

10.4 Procedural Specific Chemical Hazards. The phenolphthalin solution contains the following:

- Ethyl alcohol can be hazardous. Wear appropriate protective clothing and eyewear when handling ethyl alcohol. Be careful not to expose face or hands to splashes.
- Sodium Hydroxide can be hazardous. Wear appropriate protective clothing and eyewear; be careful not to expose face or hands to splashes. A rapid release of heat can be produced when dissolving sodium hydroxide pellets.
- Zinc is flammable. Avoid exposure to open flames or sparks.

11 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Camps, F.E., editor. *Gradwohl's Legal Medicine*. Baltimore: Williams and Wilkins (1968).

Gaensslen, R.E. *Sourcebook in forensic serology, immunology, and biochemistry*. U.S. Department of Justice, National Institute of Justice, Washington, D.C. (1983).

Lee, H. C. Identification and grouping of bloodstains. Saferstein, R., ed., In: *Forensic Science Handbook*, Prentice-Hall, 267-337 (1982).

Rev. #	Issue Date	History
7	05/25/16	<p>Changed nDNAU to DCU or BAU and updated references to DNA procedures.</p> <p>Simplified entire procedure.</p> <p>2 and 3: KP will be a dilute blood sample. Testing by biologists has demonstrated up to 1:250 consistently yielded positive results.</p> <p>Removed reagent preparation info now contained in QA SOP.</p> <p>Moved reagent QC to Section 6.</p> <p>4: Added information to sample selection.</p> <p>5.5: Removed FT POS result in 10-15 second option.</p> <p>9: Revised limitations section.</p> <p>10.4: Added zinc hazards</p>
8	02/03/20	<p>1 Updated scope</p> <p>2 Updated swab supplier</p> <p>3 Added option to test neat blood</p> <p>5.3.3 Revised to unexpected and added note for expected color change with heavily saturated blood stains</p> <p>Changed phenolphthalein to phenolphthalin throughout.</p>

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 01/31/2020

DCU Chief

Date: 01/31/2020

Procedures for the Confirmatory Identification of Blood

1 Scope

These procedures describe the method by which blood is conclusively identified on evidentiary items submitted to the FBI Laboratory for examination by the DNA Casework unit (DCU) or Biometrics Analysis Unit (BAU) using the Takayama hemochromogen (aka Taky) test.

2 Equipment/Materials/Reagents

- Takayama hemochromogen reagent
- Known positive blood control sample
- Glass microscope slides, 1" x 3" (Fisher Scientific, 12-544-1, or equivalent)
- Glass microscope slide coverslips (Fisher Scientific, 12-542A, or equivalent)
- Fume Hood (Fisher Hamilton, SAFEAIRE®, 54L0335, or equivalent)
- Stirrer/Hot Plate (Corning Model PC-220, or equivalent)
- Microscope with lenses for 100x magnification (Leitz, Laborlux 11, or equivalent)
- General laboratory equipment and supplies (e.g., scalpel, forceps)

Refer to the appropriate DNA QA procedure for reagent and control preparation information.

3 Standards and Controls

The known positive (KP) blood control is a dried human blood sample. The known negative (KN) blood control is a portion of unstained cotton sheeting or swab. The Takayama hemochromogen reagent must be tested on a KP and KN prior to first daily use on evidentiary items to verify the continued detection efficacy. A bottle of Takayama hemochromogen reagent that does not yield a positive reaction with a KP blood control, or that yields a positive reaction with a KN blood control, must not be used for casework.

4 Sample Selection

4.1 Items with an indication that blood may be present (e.g., phenolphthalein positive red/brown staining, scenario information) may be tested using the Takayama hemochromogen test. Generally, at least one stain on an item that tested positive using the phenolphthalein test will be tested using the Takayama hemochromogen test.

4.2 If a limited amount of biological material is observed on an item that is being tested for the presence of blood, Takayama hemochromogen testing may not be conducted on a stain(s) that has tested presumptively positive for blood. Foregoing confirmatory testing for blood on such stains ensures that as much stain material as possible is available for potential DNA testing in the Laboratory, as well as for any potential future testing that may be necessary.

4.2.1 If the presumptive blood test is positive, but the Takayama hemochromogen confirmatory test could potentially consume the majority of a stain, the Takayama hemochromogen confirmatory test must not be conducted. This circumstance is recorded in the case notes as quantity not sufficient (or QNS).

4.2.2 If the presumptive blood test is positive, but circumstances other than the physical characteristics of a stain (i.e., non-probative item, multiple blood stains, the potential for latent print examinations, etc.) limit the potential value of any additional consumptive testing; the Takayama hemochromogen confirmatory test may not be conducted. This circumstance is recorded in the case notes as not further characterized (or NFC).

5 Procedures

Refer to DNA Procedures Introduction (DNA QA 600) for applicable laboratory quality assurance and cleaning instructions.

Ensure the appropriate fields (i.e., reagents, KP) in STACS are completed from any network computer, as necessary.

5.1	Place a small portion (e.g., an approximately 2 mm length of textile fiber, a visible amount of scraping, an approximately 1 mm ² fabric cutting, etc.) of the questioned stain on a glass microscope slide.	
5.2	Cover the sample with a piece of coverslip that closely matches the size of the sample.	
5.3	Place the slide on a warm hot plate (generally at a heat setting of 3) in a fume hood. Place ~1-2 drops of Takayama Hemochromogen reagent sufficiently close to the coverslip piece so that some of the solution is drawn under the coverslip and into contact with the sample. Warm the solution on the slide (generally for 30 to 60 seconds).	

If necessary, additional Takayama Hemochromogen reagent may be added to the slide to prevent the sample from evaporating to dryness.

The slide may be chilled to facilitate crystal formation. Generally less than 10 minutes in a refrigerator or less than 60 seconds in a freezer is sufficient. Slides may be left in a refrigerator overnight prior to viewing.

5.4	View the slide under a microscope (generally at 100x magnification or higher if necessary).	
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5.4.1	<p>The observation of characteristic red or salmon-pink, feathery and branched, rhomboid crystals (See Figure 1) is recorded in the case notes as a positive result (or POS).</p> <div data-bbox="532 359 1097 520" data-label="Image"> </div> <p>Figure 1: Pyridineferroprotoporphyrin Crystals</p> <p>The identification of crystals must be confirmed by a serology qualified examiner. The examiner will be recorded in the case notes.</p>	
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5.4.2	<p>The observation of no characteristic crystals is recorded in the case notes as a negative result (or NEG).</p>	
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If the Takayama hemochromogen test yields a negative result, the test may be attempted additional times provided that these attempts do not consume the stain material. When the test is repeated, a notation of the number of times the test was performed must be made in the examination notes. Generally, the test will be performed either one or two times depending on the stain.

5.4.3	<p>The observation of crystals of any other color and/or morphology is recorded in the case notes as an inconclusive result (or INC).</p>	
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The formation/presence of non-Hemochromogen crystals may consume the Takayama Hemochromogen reagent (making it unavailable for reaction with any heme groups present) or potentially mask or alter any potential Hemochromogen crystals that may be present. If such an observation is made, an Examiner should be consulted prior to conducting any additional testing.

The language an Examiner should use to report the test results from this testing and others is contained within the appropriate procedure (i.e., Sero 100) in the *DNA Procedures Manual*.

6 Quality Control Procedures

6.1 Each new batch of Takayama hemochromogen reagent will be tested for efficacy at the time of its preparation using the analytical procedures above on a KP blood control and against a KN blood control.

6.1.1 A positive test result (i.e., characteristic crystals) for the KP blood control establishes that the new batch of Takayama hemochromogen reagent is yielding the expected positive result for dried human blood. A new batch of Takayama hemochromogen reagent that does not yield a positive reaction with a KP blood control is not assigned a unique identifier (i.e., batch number or barcode) and must not be used for casework.

6.1.2 A negative test result (i.e., no characteristic crystals) for the KN blood control establishes that the new batch of Takayama hemochromogen reagent is not itself yielding a positive result. A new batch of Takayama hemochromogen reagent that yields a positive reaction with a KN blood control is not assigned a unique identifier (i.e., batch number or barcode) and must not be used for casework.

6.2 If the expected results for both the KP and KN blood controls are obtained using the new batch of Takayama hemochromogen reagent, that preparation of Takayama hemochromogen reagent may be assigned a unique identifier (i.e., batch number or barcode) and may be used for casework.

7 Calculations

Not applicable.

8 Measurement Uncertainty

Not applicable.

9 Limitations

9.1 While a negative Takayama hemochromogen test indicates that no blood was detected in a stain, the failure to detect blood in biological material is not the basis for a conclusive determination that blood is not present. False-negative test results (i.e., no characteristic crystals when blood is present) may be obtained in the presence of blood when it is present in a quantity below the detection limit of the Takayama hemochromogen test.

9.1.1 A false-negative test result may be obtained from a stain that fails to efficiently solubilize into the Takayama hemochromogen reagent (e.g., generally older stains that have dried to the point of having lost their layer of molecular hydration). The temperatures and incubation times used in these procedures were optimized to facilitate pyridineferroprotoporphyrin crystal formation in the types of bloodstains encountered in forensic casework.

9.1.2 The sensitivity (i.e., detection limit) of the Takayama hemochromogen test procedure described here has not been empirically determined in the Laboratory; however, based on the nature of the test, this procedure is expected to be less sensitive than the phenolphthalein test.

10 Safety

10.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. All DNA personnel who work with such material will follow the

“Bloodborne Pathogen (BBP) Exposure Control Plan (ECP)” found in the most current version of the *FBI Laboratory Safety Manual*.

10.2 Refer to “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual* for important personal safety information prior to conducting these procedures.

10.3 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated. Unused Takayama hemochromogen reagent, as well as slides that contain the reagent, must be handled and disposed of as a regulated hazardous material.

10.4 Procedural Specific Chemical Hazard: Sodium Hydroxide can be hazardous. Wear appropriate protective clothing and eyewear; be careful not to expose face or hands to splashes. A rapid release of heat can be produced when dissolving sodium hydroxide pellets.

11 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Camps, F.E., editor. *Gradwohl's Legal Medicine*. Baltimore: Williams and Wilkins, 1968.

Gaensslen, R.E. *Sourcebook in Forensic Serology, Immunology, and Biochemistry*. U.S. Department of Justice, National Institute of Justice, Washington, D.C., 1983.

Hatch, A.L., A Modified Reagent for the Confirmation of Blood, *Journal of Forensic Sciences*, (1993) 38(6):1502-1506.

Lee, H. C. Identification and grouping of bloodstains. Saferstein, R., ed., In: *Forensic Science Handbook*, Prentice-Hall, 1982; 267-337.

Rev. #	Issue Date	History
5	04/24/13	<p>Changed “pink, feathery and branched, rhomboid crystals” to “characteristic crystals” and “filter purified” water to “reagent grade” water throughout document.</p> <p>3: Removed equipment: autoclave, filter purified water, bleach. Changed “Hotplate/stirrer” to “Stirrer/Hot Plate”. Added purchased water throughout.</p> <p>4.1: Changed “lot” to “batch” throughout.</p> <p>4.1.1: Removed directions to make KP. User is directed to SOP 106.</p> <p>7.1: Updated reference to SOP 100.</p> <p>7.2.2: Revised for clarification and added a footnote #1 to remind the user that the KP used to evaluate the Takayama reagent may be different from the one used to evaluate the Phenolphthalin reagent.</p> <p>7.3: Footnotes regarding glass containers, expiration information, and autoclaving were deleted and renumbered remaining footnotes. Added that volumes can be adjusted and that purchased reagents may be substituted.</p> <p>7.3.1: Removed directions to make 10% bleach solution. Renumbered remaining sections.</p> <p>7.3.2: Revised for consistency with SOP 200.</p> <p>7.3.4: Removed directions to make cotton swatches and directed the user to SOP 117.</p> <p>7.4.3: Removed “slide warmer” since a hot plate is usually used.</p> <p>7.4.6: Added “characteristic red or salmon-pink”.</p> <p>7.4.8: Added that an FE should be consulted for an INC result.</p> <p>7.4.9: Added “generally twice”.</p> <p>7.5: All reporting procedures were removed from this SOP. The user is directed to SOP 100 for reporting language.</p> <p>Footnote #8 of 10.1: Updated reference in footnote to SOP 106.</p> <p>10.2.1: Changed “consume a stain in its entirety” to “consume the majority of a stain”.</p> <p>11.3: Added that slides with Takayama reagent also disposed with hazardous material.</p> <p>12: Deleted Miscellaneous Procedures Manual reference.</p>
6	05/25/16	<p>Removed nDNAU throughout. Changed to DCU. Added BAU. Updated references to DNA SOPs.</p> <p>Simplified entire procedure.</p> <p>Added sample selection information.</p> <p>Relocated 4.2 through 4.2.2 from limitations.</p> <p>Relocated reagent QC to end of procedure.</p> <p>9.1.1: Moved from footnote.</p>

Approval

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Procedure for the Christmas Tree Stain to Identify Sperm Cells

1 Scope

These procedures apply to DNA personnel who create slides stains on evidentiary items or samples undergoing differential extraction, and stain slides using Kernechtrot-picroindigocarmine (i.e., Christmas Tree Stain) to aid in the microscopic identification of sperm cells.

2 Equipment/Materials/Reagents

Equipment/Materials

- General laboratory supplies (e.g., pipettes, scalpel, tubes)
- Masked glass microscope slides, 3" x 1" (Tekdon Slide #516-051-120, or equivalent)
- Adhesive gaskets (Independent Forensics of Illinois, 8005, or equivalent)
- Coverslips (vWR, 48366 205, or equivalent)
- Hot plate (Corning Model PC-220, or equivalent)
- Microscope (Olympus CX31, or equivalent, 200x or 400x magnification)
- Costar® spin baskets, or equivalent

Reagents

- SERI Christmas Tree (Xmas Tree) Stains A and B (SERI, R540, or equivalent)
- Ethanol, 95%
- Water, Reagent Grade or equivalent
- Permout (Fisher Scientific, S70104, or equivalent)

3 Standards and Controls

A known positive (KP) control (dried human semen slide) must be processed in parallel with each staining batch, and should be the first slide examined prior to the examination of evidence slides. The KP control is used to demonstrate that the staining procedure was successful and is a reference for the expected staining and morphology of a sperm cell. If the KP control fails, no conclusions can be made for the samples in the staining batch. No control is required for slides that are prepared but are not stained or microscopically examined.

4 Sample Selection

Items with an indication that semen may be present (e.g., acid phosphatase positive, scenario information) are appropriate for slide preparation and/or microscopic examination.

5 Procedures

Refer to the appropriate DNA procedure (DNA QA 600) and follow applicable general precautions and cleaning instructions.

Slide may be prepared independent of staining and microscopy and may be performed by different individuals.

If necessary, apply a frame gasket to a submitted smear slide and proceed to staining. If a proper seal cannot be attained, the procedure may be continued without a gasket.

5.1 Slide Preparation

If another slide preparation method is used, record the details in the case notes and proceed to staining.

5.1.1 From Male Fraction of Differential Extraction

5.1.1.1	After the QIAcube “Separate and Lyse 12B Mod” protocol is run, the tubes containing the washed male fraction should be resuspended (generally by vortexing and a brief quick-spin).	
5.1.1.2	Remove a portion of the washed male fraction (generally 4 µL from each sample tube) and pipette it onto a labeled microscope slide (generally a masked slide is used).	
5.1.1.3	Allow the slide(s) to dry (generally 10 minutes) and then proceed to staining or store the slide for future staining.	

5.1.2 From a Possible Semen Stain (without a DNA extraction)

5.1.2.1	Place ~1.5 mm x 1.5 mm of a stain or equivalent cutting of a swab in a tube. Add 500 µL of water. (More or less may be used to cover cutting.) Incubate at room temperature for ~30 minutes.	
5.1.2.2	Vortex and pulse spin. Transfer the cutting to a spin basket*. Spin tubes (generally between 9,000 and 13,000 rpm for 5 minutes).	

*Lyse & Spin baskets must NOT be used.

5.1.2.3	Pipette 10 µL from the area of potential pelleting onto a labeled microscope slide.	
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5.1.2.4	Allow the slide(s) to dry (generally 10 minutes) and then proceed to staining or store the slide for future staining.	
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5.2 Staining

Ensure the appropriate fields (i.e., reagents, KP) in the Sample Tracking and Control System (STACS) are completed from any network computer, as necessary.

5.2.1	Fix the slides by placing on a hot plate set at 3 for approximately 15 seconds.	
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5.2.2	Add Christmas Tree Stain A (red reagent) to cover the specimen area being stained (generally 2-3 drops per masked slide or 10-15 drops per full slide). Incubate on a level surface at room temperature for 10-15 minutes.	
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5.2.3	Gently rinse slide with reagent grade water and appropriately discard waste.	
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Generally ~1 mL is sufficient for rinsing a masked slide and ~1-3 mL for a full slide.

5.2.4	Add Christmas Tree Stain B (green reagent) to cover the specimen area being stained (generally 2-3 drops per masked slide or 10-15 drops per full slide). Incubate on a level surface at room temperature for ~15 seconds.	
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Staining one slide at a time with Stain B will facilitate keeping the incubation time less than 15 seconds. Greatly exceeding the ~15 second incubation time may lessen the intensity of the red stain and should be avoided.

5.2.5	Gently rinse slide with 95% ethanol and collect rinsate in an appropriate waste container. Allow slide to dry at room temperature (generally ~5 minutes).	
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Generally ~1 mL is sufficient for rinsing a masked slide and ~1-3 mL for a full slide.

5.2.6	In a chemical fume hood, add Permout (generally 1-2 drops for a masked slide or 10-15 drops for a full slide) and an appropriately-sized coverslip to the stained area. Permout will dry in ~10 minutes but slides may be viewed before completely dry.	
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5.3 Microscopy

5.3.1	Scan the stained portion of the slide(s) at an appropriate magnification (generally 200x or 400x) and using appropriate condenser settings (for example, brightfield or "0", phase 1 "Ph1", and/or phase 2 "Ph2") for any cellular material exhibiting suitable sperm head morphology. Sperm heads are generally dark pink/red with a pale pink/white acrosomal cap. If the	
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	sperm cell has an intact tail, it may be stained a faint green color. Epithelial cells are typically blue/green with large red/purple nuclei.	
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5.3.2	When an intact sperm cell(s) or an appropriately stained, morphologically suitable sperm head(s) is observed, record a positive result (or POS) in the casework notes.	
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5.3.3	When no morphologically suitable sperm heads or intact sperm cells are observed, record a negative result (or NEG) in the casework notes.	
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The confirmation of a sperm cell or sperm head must be made by a serology qualified Examiner. The confirming Examiner will be recorded in the casework notes.

The language an Examiner should use to report the test results from this examination and others is contained within the appropriate procedure (i.e., Sero 100) in the *DNA Procedures Manual*.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 Insufficient stain quantity and/or quality may affect the number of sperm cells observed during a smear slide examination.

8.2 While the absence of sperm cells indicates that no semen was detected on an item, the failure to detect sperm cells in biological material is not the basis of a conclusive determination that semen is not present.

8.3 Because the cellular structure of a sperm cell is susceptible to damage from the uncontrolled conditions to which a semen stain may be exposed between the time of its deposition and the time of its collection, the sensitivity of microscopically based methods of semen detection may be reduced for a particular specimen.

8.4 Though this staining procedure is not human specific (i.e., other mammalian sperm cells will also be differentially stained), human sperm have distinct morphological characteristics that allow for their identification.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Procedural Specific Chemical Hazards:

- Ethanol can be hazardous. Wear appropriate protective clothing and eyewear when handling. Be careful not to expose face or hands to splashes.
- Stain A contains aluminum sulfate which can be harmful if inhaled, if comes into contact with skin, or if swallowed. Wear appropriate protective clothing and eyewear when handling.
- Stain B contains picric acid. This staining procedure uses a dye prepared in a saturated solution of picric acid. As a dry material, picric acid is an impact and/or shock sensitive explosive. Do not allow the liquid picric acid reagent to collect and dry between the cap and lid of the container. Do not open the container when dried material appears to be present, and if present contact the Laboratory Health and Safety Group.
- Permunt Mounting Media should be used in a chemical fume hood. It is flammable and may cause central nervous system depression. It is an aspiration hazard, can enter lungs and cause damage, and may be absorbed through intact skin.

10 References

DNA Procedures Manual

FBI Laboratory Quality Assurance Manual

FBI Laboratory Safety Manual

Allery JP, Telman N, Mieusset R, Blanc A, Rough D. Cytological detection of spermatozoa: comparison of three staining methods. *J Forensic Sci* 2001; 46(2):349-351.

Leubitz SS, Savage RA. Sensitivity of Picroindigocarmine/Nuclear Fast red (PIC/NF) Stain for the Detection of Spermatozoa: A Serial Dilution Study of Human Ejaculate. *Am J Clin Pathol* 1984; 81: 90-93.

Oppitz E. A New Method of Dyeing Used to Detect Traces of Spermatozoa in Sexual Offenses.
Arch Kiminol 1969; 144: 145-148.

Serological Research Institute. Christmas Tree Stain R540 Informational Flyer, November 2011.

Rev. #	Issue Date	History
1	09/10/14	Entire document revised to reflect new practice.
2	05/25/16	Updated references to DNA Procedures Manual 1: Added slide preparation. 2: Added Costar basket. 3: KP is processed with each staining batch. Added purpose of KP. No control needed for slides not stained. 4: Relocated and renamed to Sample Selection and added guidance. 5.1.2: Added slide prep from stain. 5.2: Changed to STACS. 5.2.3: Added appropriately. 5.2.6: Changed to 1-2 drops. 9.3: Added Stain A hazard info. Removed confirmation and reporting from procedural step boxes.
3	03/22/19	1: Updated scope 2: Removed no longer available Spray-Cyte™ Fixative, added hot plate. 3: Added if the KP fails 5.1.1.2: Added from each tube to encompass samples that will be combined. 5.2.1: Replaces Spray-Cyte™ Fixative instruction with heat fixing instruction. 9.3: Removed Spray-Cyte™ hazards

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Approval

DNA Technical Leader

Date: 03/21/2019

DCU Chief

Date: 03/21/2019

QA Approval

Quality Manager:

Date: 03/21/2019

Procedures for the One-Step Acid Phosphatase Spot Test

1 Scope

These procedures describe the method in the DNA Casework Unit (DCU) by which evidentiary items are screened for the possible presence of semen by testing for acid phosphatase (AP) using the One-Step AP Spot Test.

2 Equipment/Materials/Reagents

- Acid Phosphatase Spot Test [Serological Research Institute (SERI) Item # R558, or equivalent]
- Culture tubes, 12 x 75 mm (Kimble Glass, Inc., # 73500 1275, or equivalent)
- General laboratory equipment and supplies (e.g., scissors, forceps, scalpel blades, rulers, tape, towels, gloves, etc.)
- Transfer pipettes (Samco Scientific Corporation, Cat. No. 232-15, or equivalent)
- Water (Reagent Grade, VWR, Catalog # 48218-710, or equivalent)

3 Standards and Controls

The AP Spot Test solution must be tested prior to its first daily use on evidentiary items to verify its continued detection efficacy.

A known positive (KP) semen control is a dried human semen sample. A known negative (KN) semen control is a clean swab. A KP and KN must be tested by each biologist, each day, prior to using an aliquot of AP Spot Test solution for casework. Aliquot(s) of AP Spot Test solution that do not yield a positive reaction (i.e., a pink to purple color) with a KP semen control must not be used for casework. Aliquot(s) of AP Spot Test solution that yield a positive reaction (i.e., a pink to purple color) with a KN semen control must not be used for casework.

4 Procedures

Refer to DNA Procedure Introduction (DNA QA 600) for applicable laboratory quality assurance and cleaning instructions.

The unique identifier [e.g., batch number (and expiration date) or barcode] of the AP Spot Test solution and KP control used for the examination of evidence must be documented in the casework notes.

4.1	Using a new, clean swab moistened to dampness with reagent grade water, rub the stained area until a visible amount of stain has been transferred to the swab, or the swab appears matted.	
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A liquid sample (e.g., vaginal aspirate, vaginal wash, etc.) may be AP tested by applying 100µL onto a clean, dry swab following centrifugation.

Swabbings from multiple stains may be collected at a given time. The biologist may collect the swabs by placing each swab into its own 12 mm x 75 mm culture tube. The culture tubes should be labeled and/or positioned in a rack to allow for identification of each swab.

Generally, samples from a single item may be collected together. Additionally, samples from an individual's sexual assault evidence collection kit may be processed together, provided that only a single evidence item is out at a time for the collection of the samples.

4.2	Add ~3-4 drops of AP Spot Test solution to each swab tip over a receptacle.	
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Excess AP Spot Test solution should be captured in a weigh boat or other receptacle. Excess liquid must be collected into an appropriate chemical waste container for disposal.

Multiple swabs may be processed concurrently in succession.

4.3	Observe the swab tip for any color change within approximately 2 minutes and record the result as indicated below: The observation of a pink to purple color POS The observation of a faint pink color Ft. POS The observation of no pink to purple color ... NEG The observation of a non-pink/purple color ... INC	
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The observation of a non-pink/purple color could mask any potential pink/purple color that would result from the presence of semen. If such an observation is made, an Examiner should be consulted prior to conducting any additional testing.

5 Reporting Procedures

Refer to SOP 100 for reporting language to use based on the test results from this analysis and others.

6 Reagent Quality Control

6.1 Each new batch of AP Spot Test solution is tested for efficacy at the time of its preparation using the above procedure on an in-use KP semen control and a KN semen control.

6.1.2 A positive test result (i.e., a pink to purple color) for the KP semen control establishes that the new batch of AP Spot Test solution is yielding the expected positive result for semen (i.e., a pink to purple color). A new batch of AP Spot Test solution that does not yield a positive reaction with a KP semen control is not assigned a unique identifier (batch number or barcode) and must not be used for casework.

6.1.2 A negative test result (i.e., no pink to purple color) for the KN semen control establishes that the new batch of AP Spot Test solution itself is not yielding a positive result (i.e., a pink to purple color) in the absence of semen. A new batch of AP Spot Test solution that yields a false-positive reaction (i.e., a pink to purple color) with a KN semen control is not assigned a new unique identifier (batch number or barcode) and must not be used for casework.

6.2 If the expected results for both the KP and KN semen controls are obtained using the new batch of AP Spot Test solution, that preparation of AP Spot Test solution may be assigned a unique identifier (batch number or barcode) and may be used for casework.

7 Sampling

Not applicable.

8 Calculations

Not applicable.

9 Measurement Uncertainty

Not applicable.

10 Limitations

10.1 A positive result (i.e., a pink to purple color) with the AP Spot Test solution provides a presumptive indication that semen may be present on an item but it does not constitute an identification of semen. A confirmatory testing procedure is required to identify the presence of semen in a questioned stain.

10.2 A faint positive result indicates that semen may be present on an item but it does not constitute an identification of semen. The faint nature of the positive result is recorded in the case work documentation to capture semi-quantitative information that may be of value in determining what, if any, additional tests are conducted on a stain (e.g., confirmatory testing, DNA testing, etc.) and/or the amount of stain consumed for such a test(s).

10.3 While a negative AP Spot Test indicates that no semen was detected in a stain, the failure to detect semen in biological material is not the basis for an absolute determination that semen was not present. False-negative test results (i.e., no pink to purple color) may be obtained when semen is present in a quantity below the detection limit of the AP Spot Test (e.g., insufficient quantity and/or poor quality). The sensitivity (i.e., detection limit) of the AP Spot Test procedure described in this document has been empirically determined in the Laboratory to generally yield a positive result from a dried stain of semen diluted 1/50.

10.4 The utility of the AP Spot Test lies in its ability to provide information that aids in the differentiation between those stains that most certainly do not contain semen and those that may so that any further testing (i.e., confirmatory testing for semen, DNA testing, etc.) can be focused on those stains most likely to yield additional information.

10.5 Presumptive semen testing may not be conducted on items of evidence of potential value for latent fingerprint examination.

11 Safety

11.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. All nDNAU personnel who work with such material will follow the “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP)” found in the most current version of the *FBI Laboratory Safety Manual*.

11.2 Refer to the “Safe Work Practices Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual* for important personal safety information prior to conducting these procedures.

11.3 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as

well as the biohazardous wastes generated.

11.4 Procedural Specific Chemical Hazard: The AP Spot Test Powder can be hazardous and may cause cancer. It may be irritating to eyes, respiratory system, and skin; it is corrosive and may cause burns.

12 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Laux, D.S., Forensic Detection of Semen I. The Acid Phosphatase Test

Rev. #	Issue Date	History
0	10/02/12	Original document issued.
1	12/01/15	Changed to nDNAU to DCU. Simplified title and text of procedure. Deleted background section. Eliminated specific general laboratory supplies from list. Removed reagent and control preparation now contained in QA SOPs. Moved reagent QC to end. Loosened language to allow ~3-4 drops. Removed interpretation information from procedural section, still described in limitations section. Relocated footnote into 10.3. Updated reference for DNA Procedures Manual

Approval

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Procedures for the Collection of Biological Material

1 Scope

These procedures apply to DNA personnel that collect samples from various types of evidentiary items that will undergo DNA analysis by the DNA Casework Unit (DCU) and Biometric Analysis Unit (BAU). Because it is not practical to anticipate all items of evidence received, or their condition, these procedures are put forth as guidelines. If an evidentiary item is encountered that is not specifically cited, samples should be collected using the procedures for the item most similar in composition and/or nature. Additional guidance may be included in substrate-specific DNA procedures (e.g., calcified tissue samples, hair). Any collection conducted in a manner not consistent with the following guidelines must be described in the case notes.

2 Equipment/Materials/Reagents

- General laboratory supplies (e.g., pipettes, scalpel, ruler)
- Sample Tubes
- Sterile Applicators (swabs)
- Water, Reagent Grade or equivalent
- Ethyl Alcohol (i.e., ethanol), 95%
- Xylene
- Alternate light source (Polilight Flare Plus or equivalent)
- Tissue homogenizer (Dounce tissue grinder or equivalent)
- Fabric swatch (cotton sheeting or equivalent)

3 Procedures

Refer to the DNA procedures introduction (i.e., DNA QA 600) and follow applicable general precautions and cleaning instructions.

3.1	For each item examined, an indication of the packaging condition (e.g., properly sealed envelope) and a description of the item(s) and the sample(s) collected from the item, if appropriate, will be captured in the case notes. -The description of the item should be enough to distinguish it from other items in the case. -The description of the collection should be enough to allow another biologist or an examiner to determine approximately where the sample was collected from.	
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If packaging and/or an item was previously described (e.g., during serology examinations), the description should be reviewed and any discrepancies or updates noted.

Multiple samples may be combined into a single tube, as appropriate.

3.2 Preparation of Dried Blood Sample from Liquid Blood

A dried blood sample may be prepared from a liquid blood sample. If a liquid blood sample is clotted, the blood should be homogenized and a dried blood sample prepared from the homogenized blood prior to a collection. Recovery from liquid blood should not be attempted for a clotted sample.

3.2.1	Label a fabric swatch with the Laboratory number, item number, name of the individual from whom the blood is identified as having been collected, the date of swatch preparation, and the initials of the individual preparing the bloodstain. Record the type of blood tube used to prepare the exemplar (e.g., purple top tube) in the case notes.	
3.2.2	Gently invert the blood tube until the contents are thoroughly mixed. Record the fill-level of the tube by marking its meniscus on the outside of the tube.	
3.2.2.1	<i>If the blood is coagulated:</i> Slowly transfer the clotted blood into the reservoir of an autoclaved tissue homogenizer. Insert the glass pestle into the reservoir and gently homogenize the clotted material and serum.	
3.2.3	In a hood, slowly pipette approximately 500 µL of liquid blood onto the fabric swatch. If the liquid blood tube contains limited volume, a smaller volume of blood should be applied to the swatch. Record the volume of blood used to prepare the stain in the case notes.	
3.2.3.1	<i>If the blood was homogenized:</i> Return the unused homogenate to the original blood tube. Ensure the glass homogenizer and pestle are rinsed with 10% bleach solution, washed, and rinsed with reagent grade water.	
3.2.4	Allow the bloodstain to thoroughly air dry within the hood (approximately 1 hour). Place the dried stain in a labeled coin envelope and store refrigerated.	

Multiple samples may be dried in the same hood provided they are sufficiently far apart to prevent their touching each other during drying.

3.3 Cutting Dried Stains (e.g., blood stains, semen stains, sexual assault kit swabs, buccal swabs, other evidentiary swabs)

3.3.1	Cut ~5 mm x 5 mm from the dried stain or ~ 1/3 to 1/2 of swab head and place it into the appropriate type of labeled tube.	
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For sexual assault kit swabs, typically ~ 3/4 of each swab from an orifice is cut and the cuttings from up to 2 swabs are placed in a single tube for extraction.

3.4 Swabbing Dried Stains (e.g., blood stains, semen stains)

3.4.1	Using a sterile swab moistened with reagent grade water, swab a sufficient portion of the stain.	
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3.4.2	Remove the entire swab head (or a portion of the swab head) and place it into the appropriate type of labeled tube.	
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3.5 Recovery from Liquid Blood

3.5.1	Gently invert the blood tube until the contents are thoroughly mixed.	
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3.5.2	Transfer an appropriate amount of blood (generally ~ 5 µL) into the appropriate type of labeled tube.	
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3.4.3	Close and tape seal the tube, and initial the tape seal.	
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3.6 Recovery from Moisture-Activated Envelope Flaps or Stamps

3.6.1	Carefully open any sealed envelope flap and/or remove any affixed stamp as appropriate using steam generated from water boiled in a household teapot.	
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Purified water available at laboratory sinks may be used for steaming.

3.6.2	Using a sterile swab moistened with reagent grade water, swab the adhesive area of flap and the corresponding region on the envelope.	
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3.6.3	Remove the entire swab head and place into the appropriate type of labeled tube.	
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3.7 Recovery from Cigarette Butts

3.7.1	Remove ~5 mm from the filter end of the cigarette butt and place it into the appropriate type of labeled tube.	
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3.8 Recovery from Bottles, Cans, Cups

3.8.1	Using a sterile swab moistened with reagent grade water, swab the mouth of the item.	
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If a dry residue may contain inhibitors (e.g., coffee, cola), this residue should be collected on a separate swab and processed as a separate sample.

If fluid remains in the container, the inside surfaces of the container should not be sampled. The liquid volume in such a container may be collected and processed as a liquid rinse or wash.

3.8.2	Remove the entire swab head and place into the appropriate type of labeled tube.	
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3.9 Recovery from Tape

3.9.1 By Swabbing

3.9.1.1	Using a sterile swab moistened with reagent grade water, swab the tape where appropriate.	
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3.9.1.2	Remove the entire swab head and place it into the appropriate type of labeled tube.	
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3.9.2 By Cutting

Tape may be adhered to a plastic sheet for the preservation of latent prints. Collection can occur after latent processing.

3.9.2.1	Take an ~1 inch cutting from the appropriate area(s) (e.g., from the unexposed end) of the tape.	
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3.9.2.2	Place the cutting with adhesive side facing out into the appropriate type of labeled tube.	
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3.10 Recovery from Clothing Items

3.10.1 Wearer Swabbing

3.10.1.1	Using a sterile swab moistened with reagent grade water, swab the contact areas of the item (e.g., the sweatband of baseball style cap; the inside collar, cuffs, and/or underarm areas of a shirt; the region around the eye, nose, and/or mouth holes of a mask).	
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Should such areas not be identifiable, a general swabbing may be taken. Begin in areas likely to have less DNA and end in areas likely to have more DNA.

Alternatively, fabric cuttings from one or more of these areas may be taken and processed.

3.10.1.2	Remove the entire swab head and place it into the appropriate type of labeled tube.	
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3.10.2 Male Underwear in Sexual Assault Scenarios

3.10.2.1	View the underwear with the alternate light source. Circle areas of fluorescence with a dotted line.	
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A photograph should be taken of the item.

3.10.2.2	Using a sterile swab moistened with reagent grade water, swab the inside front area(s) of the underwear circled with a dotted line.	
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3.10.2.3	Remove the entire swab head and place it into the appropriate type of labeled tube.	
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3.10.2.4	Using a sterile swab moistened with reagent grade water, swab the outside front area(s) of the underwear circled with a dotted line.	
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3.10.2.5	Remove the entire swab head and place it into the appropriate type of labeled tube.	
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Should such areas not display fluorescence, a general swabbing of the inside front of the underwear and a general swabbing of the outside front of the underwear may be taken.

3.11 Recovery of Handler DNA from Firearms, Knives, and Weapons

3.11.1	Using a sterile swab moistened with reagent grade water, swab those portions of the weapon that would most likely have been handled by the individual (e.g., the handle of the knife; the textured portions of the grip, trigger, or hammer of the firearm).	
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3.11.2	Remove the entire swab head and place it into the appropriate type of labeled tube.	
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3.12 Recovery from Fingernail Clippings

3.12.1	Using a sterile swab moistened with reagent grade water, swab the underside of all clippings. Generally, separate swabs should be taken from the clippings of each hand if submitted.	
3.12.2	Remove the entire swab head and place it into the appropriate type of labeled tube.	

3.13 Recovery from Microscope Smear Slides

3.13.1	Using a sterile swab moistened with reagent grade water, remove ~½ to ¾ of the smear from the microscope slide.	
3.13.2	Remove the entire swab head and place it into the appropriate type of labeled tube.	

3.14 Recovery from Microscope Smear Slides with Cover-Slips

3.14.1	Attempt to remove the cover-slip from the microscope slide, using a scalpel if necessary.	
3.14.2	If necessary, cover the appropriate portion of the slide in xylene until the cover slip can be removed. Alternatively, the entire slide may be soaked in xylene. Discard the liquid into an appropriately labeled container suitable for xylene waste. Allow the slide to evaporate to dryness.	

Xylene must be handled in a fume hood and the slide should be kept in the fume hood until dry.

3.14.3	Using a sterile swab moistened with reagent grade water, remove ~½ to ¾ of the smear from the slide and the cover-slip.	
3.14.4	Remove the entire swab head and place it into the appropriate type of labeled tube.	

3.15 Recovery from Liquid Rinses or Washes

3.15.1	Centrifuge the tube containing the rinse or wash in the appropriate centrifuge for 10 minutes at maximum speed.	
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If the rinse is in a tube not compatible with the centrifuge, transfer the rinse to an appropriately labeled tube prior to centrifugation and the supernatant can be transferred back to the original tube after centrifuging.

3.15.2	Transfer the supernatant from over the pelleted material into an appropriate tube.	
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3.15.3	Collect pelleted material onto a sterile swab. Remove the entire swab head and place it into the appropriate type of labeled tube.	
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3.15.4	Transfer the supernatant back into the original tube.	
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3.16 Recovery from Biological Tissue

3.16.1	Dissect $\sim 1 \text{ cm}^3$, if available, from the tissue mass and mince.	
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3.16.2	Place minced tissue into the appropriate type of labeled tube(s) so that tube(s) is $\sim \frac{1}{3}$ to $\frac{1}{2}$ full.	
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3.17 Recovery from Paraffin-Embedded Biological Tissue

3.17.1	Dissect $\sim 1 \text{ cm}^3$, if available, from the tissue mass and mince.	
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Remove any excess paraffin, if possible.

3.17.2	Place minced tissue into the appropriate type of labeled tube(s) so that tube(s) is $\sim \frac{1}{3}$ to $\frac{1}{2}$ full.	
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3.17.3	In a fume hood, add enough xylene to the tube to fully immerse the sample, vortex, and incubate for ~ 30 minutes at room temperature with agitation.	
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3.17.4	Spin to pellet (generally 9,000 to 13,000 rpm for 5 minutes).	
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3.17.5	In a fume hood, decant the supernatant into an appropriately labeled container suitable for xylene waste.	
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3.17.6	Repeat the xylene wash procedure.	
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3.17.7	Add 95% ethanol equivalent to half the volume of xylene and vortex.	
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3.17.8	Spin to pellet (generally 9,000 to 13,000 rpm for 5 minutes).	
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3.17.9	Decant the supernatant into an appropriately labeled container suitable for ethanol waste.	
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3.17.10	Repeat the ethanol wash procedure.	
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3.17.11	Allow the de-paraffinized sample to air dry at room temperature (generally for 2-4 hours) in a hood.	
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If necessary, transfer the sample to a secondary container in a hood to facilitate drying. Transfer the sample back to the labeled tube following drying.

3.18 Recovery from Aborted Tissue

3.18.1	If frozen, allow the sample to thaw at room temperature for several hours or at 4°C overnight.	
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The liquid surrounding the specimen may contain a large proportion of maternal blood. Sample may be transferred to a secondary container to facilitate collection.

3.18.2	If possible, select recognizable pieces from the tissue, transfer them to another container, and rinse with reagent grade water. Additional reagent grade water can be used to facilitate the collection process.	
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Aborted tissue may contain both embryonic/fetal and maternal tissues. Both the embryonic/fetal DNA and the maternal DNA may be isolated. Additional information can be found in Johnson, et. al. (2010).

3.18.3	Dissect ~1 cm ³ , if available, from the selected tissue and mince.	
3.18.4	Place minced tissue into the appropriate type of labeled tube(s) so that tube(s) is ~1/3 to 1/2 full.	

3.19 Recovery from Hair Samples

Refer to the DNA procedure for the extraction of DNA from hair and keratinized tissue (i.e., DNA 402) for additional collection and wash guidance for hairs to be extracted for mitochondrial DNA processing.

3.19.1 Recovery from Hair Samples to be Used as a Reference Sample

3.19.1.1	Rinse the hair thoroughly with 95% ethanol. Discard the wash into an appropriately labeled container suitable for ethanol waste.	
3.19.1.2	Follow the ethanol rinse with a thorough rinse with reagent grade water.	
3.19.1.3	Remove ~1 cm from the root end and place it into the appropriate type of labeled tube.	

3.19.2 Recovery from Hair Samples of Unknown Origin

3.19.2.1	Remove ~1 cm from the root end and place it into the appropriate type of labeled tube.	
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3.19.3 Recovery from Mounted Hair Samples

3.19.3.1	Attempt to remove the cover-slip from the microscope slide, using a scalpel if necessary.	
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3.19.3.2	If necessary, cover the appropriate portion of the slide in xylene until the cover slip can be removed. Alternatively, the entire slide may be soaked in xylene. Discard the liquid into an appropriately labeled container suitable for xylene waste.	
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Xylene must be handled in a fume hood.

3.19.3.3	Rinse the hair with ~5 mL 95% ethanol. Discard the wash into an appropriately labeled waste container suitable for ethanol waste.	
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3.19.3.4	Follow the ethanol rinse with a thorough rinse with reagent grade water.	
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3.19.3.5	Remove ~1 cm from the root end and place it into the appropriate type of labeled tube.	
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4 Sampling or Sample Selection

DNA personnel rely on knowledge, training and experience to select the appropriate samples and/or stains to test. In addition, an examiner may rely on the results of the serological testing and/or observations noted by the biologist regarding the selection of appropriate stains (e.g., dark red stain versus faint red stain, swab with yellow staining versus swab with no staining). Where information does not allow two stains/samples to be distinguished from one another (e.g., two swabs with no staining observed on either), a stain/sample may be selected at random.

5 Standards and Controls

Controls are initiated at extraction. Refer to the appropriate DNA extraction procedure for the introduction of the appropriate controls.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

These procedures do not exhaust the possible list of evidentiary items that may be encountered. For those items not specifically cited, samples should be collected using the procedures most similar in composition and/or nature.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Procedural Specific Chemical Hazards

- Ethyl alcohol and xylene are hazardous materials. Use only in a fume hood. Wear appropriate protective clothing and eyewear when handling both. Be careful not to expose face or hands to splashes.

10 References

FBI Laboratory Quality Assurance Manual

FBI Laboratory Operations Manual

FBI Laboratory Safety Manual

DNA Procedures Manual

An SF and Fleming KA. Removal of inhibitor(s) of the polymerase chain reaction from formalin fixed, paraffin wax embedded tissues, *Journal of Clinical Pathology* (1991) 44: 924-927.

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Impraim CC, Saiki RK, Erlich HA, and Teplitz RL. Analysis of DNA extracted from formalin-fixed, paraffin-embedded tissues by enzymatic amplification and hybridization with sequence-specific oligonucleotides. *Biochem Biophys Research Comm* (1987) 142: 710-716.

Johnson DJ, Matthies LK, Roberts KA, Yorker BC. Isolation and individualization of conceptus and maternal tissues from abortions and placentas for parentage testing in cases of rape and abandoned newborns. *Journal of Forensic Sciences* (2010) 55(6): 1430-1436.

Shimizu H and Burns JC. Extraction of nucleic acids: sample preparation from paraffin-embedded tissues. In: *PCR Strategies*. M Innis, D Gelfand and J Sninsky eds. Academic Press, NY, 1995, pp. 32-38.

Wright DK and Manos MM. Sample preparation from paraffin-embedded tissues. In: *PCR Protocols: A Guide to Methods and Applications*. MA Innis, DH Gelfand, JJ Sninsky and TJ White, eds. Academic Press, NY, 1990, pp. 153-158.

Rev. #	Issue Date	History
12	03/08/16	<p>This procedure may also be used by BAU DNA personnel.</p> <p>Minor wording edits throughout.</p> <p>Added ALS to 2.</p> <p>Added reference to evidence management and examination practices and procedures.</p> <p>Revised 3.1 to give guidance for items/sample descriptions.</p> <p>3.8.2 Added specific guidance for cutting tape.</p> <p>3.10 Specified for handler DNA.</p> <p>3.16 Reworded removing excess paraffin guidance.</p> <p>4 Reworded sample selection, applies to DNA personnel.</p>
13	02/28/18	<p>1 Reworded scope</p> <p>2 and 3.2 Added blood spotting guidance from SOP 108-5.</p> <p>Renumbered remaining sections.</p> <p>3.1 Changed info captured in case notes from should to will.</p> <p>3.3.1 Added guidance for typical SAK swab collection.</p> <p>3.6.2 and 3.8.1 Removed repeat with second swabbing.</p>

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 02/27/2018

DCU Chief

Date: 02/27/2018

BAU Chief

Date: 02/27/2018

QA Approval

Quality Manager

Date: 02/27/2018

Procedures for CODIS Entry and Upload of DNA Typing Results

1 Scope

These procedures apply to DNA personnel who enter and upload DNA profiles into the **CO**mbined **DNA** Index System (CODIS) and disposition matches in CODIS.

2 Background

CODIS is a collection of DNA databases from forensic and databasing laboratories throughout the United States. CODIS consists of various indices including a forensic index, an offender index, and a missing persons index. CODIS enables federal, state, and local DNA laboratories to exchange and compare DNA profiles in order to link crimes to one another and to offenders. In addition, it allows DNA profiles from unidentified human remains to be linked to forensic samples, offender samples, and to samples involving missing persons.

CODIS functions as three separate tiers: local, state, and national. DNA profiles originate at the local level (LDIS – Local DNA Index System) and are then uploaded to state and national levels to be searched. The SDIS (State DNA Index System) laboratory typically serves as the central point of contact for access to the National DNA Index System (NDIS). NDIS is the national, FBI-administered, centralized database of all DNA profiles contributed by SDIS laboratories. The FBI Laboratory CODIS Unit manages the NDIS database and supports the SDIS and LDIS laboratories by providing computer software, training, and assistance with searches. This tiered approach enables local, state, and federal laboratories to manage the samples in their databases based upon their specific legislative requirements.

The FBI Laboratory enters DNA profiles (i.e., STR profiles or mitochondrial DNA sequences) from forensic and missing persons' samples into LDIS. Eligible profiles are then uploaded to SDIS where they are compared to Federal DNA Database Unit (FDDU) profiles and profiles submitted by the Bureau of Alcohol, Tobacco, Firearms, and Explosives (ATF). Eligible profiles are then uploaded to NDIS where they are compared against all applicable indices. CODIS uploads and searches are conducted on a regular basis.

3 Equipment/Materials/Reagents

Combined DNA Index System (CODIS) Software (version 8.0 or higher)

4 Procedures

4.1 CODIS Specimen Identification Number

4.1.1 DNA profiles are entered into CODIS using a standardized format that is typically generated by the Sample Tracking and Control Software (STACS). The CODIS Specimen ID is a unique identifier that generally consists of the laboratory number, the item identifier and stain identifier (e.g., 2017-00349_1(1)A).

4.1.2 For differentially extracted samples from a single item where profiles from both fractions are eligible for CODIS entry, a suffix must be added in STACS, if applicable, to differentiate the samples. STACS will generate the same CODIS specimen name otherwise (e.g., 2017-00349_1(1)A). This suffix addition can designate which fraction the profile came from (e.g., 2017-00349_1(1)AF, 2017-00349_1(1)AM). For forensic mixtures that result in major/minor designation, the portion of the profile being entered into CODIS may be indicated by adding information into the suffix area in STACS, if applicable (e.g., 2017-00349_1(1)AMAJ, 2017-00349_1(1)AMIN).

4.2 Data Review Requirements Prior to CODIS Entry and Uploading

4.2.1 Each CODIS User will complete the “Annual NDIS Eligibility Training” which is required per the *National DNA Index System (NDIS) Operational Procedures Manual*.

4.2.2 All DNA profiles must undergo primary analysis and technical review by appropriately qualified DNA Examiners prior to CODIS entry. Interpretation/review of the data must be performed in accordance with the appropriate interpretation standard operating procedure (SOP) of the *DNA Procedures Manual*.

4.2.2.1 If samples are outsourced, eligible profiles must undergo technical review by an appropriately qualified DNA Examiner prior to CODIS entry.

4.2.3 The qualified DNA Examiner is responsible for determining whether or not the DNA profile is eligible for entry into CODIS and assigning the specimen to the correct specimen category.

4.2.3.1 The original 13 CODIS core loci are: D3S1358, vWA, D16S539, CSF1PO, TPOX, D8S1179, D21S11, D18S51, TH01, FGA, D5S818, D13S317 and D7S820.

4.2.3.2 Specimen categories available:

- **Forensic Unknown:** This category is for samples from a single source (or a fully deduced profile originating from a mixture) from a putative perpetrator that contains results for all of the original 13 CODIS core loci. This profile shall not have more than 3 alleles at one locus while the remaining loci can have up to 2 alleles. There should be no indication of locus or allelic dropout at the original 13 CODIS core loci. This specimen category is used for uploading to NDIS.

- **Forensic Partial:** This category is for samples originating from a single source (or a fully deduced profile originating from a mixture) from a putative perpetrator with either locus or allelic dropout at one or more of the original 13 CODIS core loci for entry into NDIS or any typed loci for entry into LDIS. This profile shall not have more than 3 alleles at one locus while the remaining loci can have up to 2 alleles. A single-source partial profile that will be entered into SDIS must be entered using the SDIS Only specimen category only. A moderate match estimate (MME) must be calculated for this specimen category.
- **Forensic Mixture:** This category is for samples that contain DNA contributed from more than one source. These profiles can contain loci with 3 or more alleles at 2 or more loci (not to exceed 4 alleles at any locus) and/or loci with 2 alleles that need to be searched at moderate stringency. This specimen category will be used for entry into NDIS (using the original 13 CODIS core loci) and LDIS (using all typed loci). If an allele at a mixed locus can be definitively attributed to the putative perpetrator, it may be designated with a plus sign “+” as an obligate allele. A MME must be calculated for this specimen category.
- **Forensic Targeted:** This category is for samples that contain DNA contributed from either a single source (or a fully deduced profile originating from a mixture) or samples that contain DNA contributed from more than one source. These profiles originate from a forensic partial or a forensic mixture that do not meet the NDIS MME threshold but do meet the match rarity estimate (MRE) threshold if searched at a specified stringency by locus (high or moderate). These profiles must contain at least 8 of the original 13 CODIS core loci. These profiles will only be searched against full single source profiles (i.e., Forensic Unknown, Convicted Offender, Arrestee, Detainee, Legal). All available loci will be searched at high stringency. Any loci with more than 2 alleles will be searched at moderate stringency. Any loci marked as partial will be searched at moderate stringency. This is considered a ‘last resort’ specimen category to upload a specimen to NDIS.
- **SDIS Only:** This category is for samples that originate from a single source (or a fully deduced profile originating from a mixture) or samples that contain DNA from more than one source that do not meet the requirement for uploading to NDIS but do meet the requirements for uploading to SDIS. Note: If a sample is being entered into LDIS or NDIS, do *not* select this category. A MME must be calculated for this specimen category.
- **Biological Child; Biological Father; Biological Mother; Sibling; Maternal Relative; Paternal Relative; Spouse:** These categories are for Relatives of Missing Persons samples that are voluntarily provided as reference samples

where amelogenin is required. All of the original 13 CODIS core loci with no drop out are required for uploading to NDIS. These samples require a signed consent form witnessed by law enforcement in order to be uploaded to CODIS.

- **Deduced Missing Person:** This category is for DNA profiles of a missing person that have been generated by examining intimate items purported to belong to the missing person (i.e. toothbrush). In order to enter these samples into CODIS, amelogenin is required and 7 of the original 13 CODIS core loci are required for upload to NDIS.
- **Missing Person:** This category is for known reference samples from an individual that is missing. The source of the DNA has been verified as originating from the missing person (e.g., tissue from a medical sample). In order to enter these samples into CODIS, amelogenin is required and 7 of the original 13 CODIS core loci are required for upload to NDIS.
- **Unidentified Human Remains (UHR):** This category is for DNA profiles from deceased individuals (e.g., bones) or an individual who is unidentified (e.g., children who can't or others who can't or refuse to identify themselves). In order to enter these samples into CODIS, amelogenin is required and 7 of the original 13 CODIS core loci are required for identity searches at NDIS.

4.2.3.3 The DNA profile from a forensic sample shall only offer those alleles that are attributed to the putative perpetrator(s). Alleles derived from forensic profiles that are unambiguously attributed to a victim or individuals other than the perpetrator(s), such as a consensual partner, will not be offered to CODIS. The qualified DNA Examiner may refer to "A Guide to Determining What is Allowable in the Forensic Index at NDIS", for clarification of what may be entered into CODIS.

4.2.3.4 The DNA results from any locus in which an ambiguity exists in the assignment of one or more alleles to the putative perpetrator(s) may be offered to CODIS. The mere observation of alleles that may be attributed to individuals other than the putative perpetrator, does not in itself preclude offering DNA profiles to CODIS at that locus.

4.2.3.5 Specimen Requirements for Forensic Samples

Tier	NDIS	SDIS	LDIS
Forensic Unknown	All original 13 CODIS core loci Tri-allele allowed @ 1 core locus Spec Cat: Forensic Unknown	NA	NA
Forensic Mixture	≥ 8 original 13 CODIS core loci ≤ 4 alleles / locus MME ≥ 10 million Obligate alleles “+” Spec Cat: Forensic Mixture	≥ 7 loci ≤ 4 alleles / locus MME ≥ 1.2 million Obligate alleles “+” Spec Cat: SDIS Only	≥ 6 loci ≤ 4 alleles / locus MME 1 in size of LDIS Obligate alleles “+” Spec Cat: Forensic Mixture
Forensic Partial	≥ 8 original 13 CODIS core loci MME ≥ 10 million Mark partial loci Spec Cat: Forensic Partial	≥ 7 loci MME ≥ 1.2 million Mark partial loci Spec Cat: SDIS Only	≥ 6 loci MME 1 in size of LDIS Mark partial loci Spec Cat: Forensic Partial
Forensic Targeted	≥ 8 original 13 CODIS core loci ≤ 4 alleles / locus MRE ≥ 10 million Obligate alleles “+” Mark partial loci Spec. Cat: Forensic Targeted	NA	NA
SDIS Only	NA	See above for Forensic Mixture, Forensic Partial	NA

4.2.4 Due to the low stringency searches among the Missing Persons, Relatives of Missing Persons, and Unidentified Human Remains Indices, profiles to be entered into these indices will not include homozygous alleles below the stochastic threshold established by the appropriate SOP of the *DNA Procedures Manual*. Profiles for entry into these indices may include heterozygous typing results below the stochastic threshold established by the appropriate interpretation SOP of the *DNA Procedures Manual*.

4.2.5 Samples for entry into the Missing Persons, Relatives of Missing Persons, and Unidentified Human Remains Indices will not include results for loci with tri-alleles. If a tri-allele is observed in an original 13 CODIS core locus, contact the casework LDIS Administrator or casework Missing Persons Program Manager for additional guidance.

4.2.6 Samples entered into the Missing Persons and Unidentified Human Remains Indices should attempt DNA typing for autosomal as well as the appropriate lineage marker(s).

4.2.7 All Relatives of Missing Persons samples must be typed for autosomal markers. In addition, at least one member of the pedigree, should be typed using an appropriate lineage marker.

4.2.8 A complete mitochondrial DNA (mtDNA) sequence includes positions 16024 to 16365 and 73 to 340 (HV1 and HV2). Profiles that meet or exceed this range are acceptable for uploading to NDIS. Profiles that are less than the NDIS definition for completeness can be stored in LDIS.

4.2.9 Profiles that have complete mtDNA sequences, but have fewer than 7 of the original 13 CODIS core loci, may have the autosomal data stored in CODIS for evaluation purposes.

4.2.10 Composite DNA profiles for forensic samples may be entered into CODIS. Uploading the maximum amount of genetic information available for a given qualifying DNA profile decreases the likelihood of spurious matches within a database the size of NDIS. To reasonably ensure that a DNA profile compiled from genetic information derived from separate extractions, amplifications, and/or injections has arisen from the same individual, the resultant DNA profile must 1) be compiled from different items from a common source (e.g., replicate vaginal swabs, swabs from the same orifice such as vaginal and cervical swabs), multiple cuttings of the same evidentiary stain, or cuttings from different stains of the same grouping on a given evidence item and 2) demonstrate concordance as defined in the appropriate interpretation SOP of the *DNA Procedures Manual*.

4.2.11 Additional questions about the suitability/eligibility of a particular DNA profile for CODIS should be directed to the casework LDIS Administrator, casework LDIS Alternate Administrator, FBI State Administrator, FBI State Alternate Administrator, or the DNA Technical Leader.

4.3 Entering and Uploading DNA Profiles into CODIS

4.3.1 Profiles eligible for entry into LDIS, SDIS, and NDIS will be entered into the appropriate specimen category (e.g., Forensic Unknown) and transferred to the appropriate index (e.g., Forensic).

4.3.2 Duplicate samples will not be marked for upload to NDIS.

4.3.3 The appropriate DNA profiles are entered into LDIS/SDIS by the casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software). Data entry and uploading of DNA profiles will be performed routinely following NDIS procedures and instructions for the current version of the CODIS software.

4.3.3.1 Profiles and/or pedigrees that are manually entered into the CODIS software should be verified by the LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software).

4.3.4 The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will review the SDIS and NDIS Reconciliation Reports to confirm the receipt and processing of the upload to SDIS and NDIS.

4.3.5 The DNA profiles at LDIS are uploaded to SDIS according to parameters set by the FBI State Administrator or the FBI State Alternate Administrator. Profiles not eligible for entry into SDIS (e.g., staff profiles) will not be marked for upload.

4.3.6 The DNA profiles at SDIS are uploaded to NDIS according to parameters set by the NDIS Custodian.

4.3.7 Datalinking may be performed by the casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software), as appropriate, following instructions for the current version of the CODIS software. DNA profile information may also be exported and provided to another laboratory for datalinking purposes.

4.4 Routine Searches of the Database

4.4.1 The CODIS software will be routinely checked for candidate matches and ranks by the casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software). A candidate match is a possible match between two or more DNA profiles discovered by the CODIS software. Ranks are potential associations between UHR samples and pedigree trees comprised of Relatives of Missing Persons samples.

4.4.2 Candidate matches and ranks will be provided to the casework LDIS Administrator (or any qualified DNA Examiner) for evaluation for moderate or low stringency hits and to the casework LDIS Administrator (or any qualified DNA Examiner or any person with administrative rights who has been trained to use the CODIS software) for evaluation for high stringency hits.

4.5 DNA Index of Special Concern (DISC) (Rapid-Enabling of Samples)

4.5.1 Qualified DNA Examiners will determine which samples meet DISC eligibility. In order to be DISC eligible, samples must be entered into the Forensic Unknown specimen category as source ID no (i.e., unsolved). These cases must be from unsolved cases of significant public safety concern. These cases of significant public safety concern are defined as sexual assault cases, homicide cases, kidnapping cases, and terrorism cases.

4.5.2 Prior to the casework LDIS administrator (or any person with administrative rights who has been trained to use the CODIS software) enrolling DISC eligible samples into the DISC, these samples must have been searched in SDIS and NDIS at least once.

4.5.3 Casework Metadata must be entered prior to unsolved, Forensic Unknown samples being enrolled into DISC. This metadata, if applicable, should be compiled by the qualified DNA Examiner. The metadata will be entered into the CODIS software by the casework LDIS administrator (or any person with administrative rights who has been trained to use the CODIS software). The required metadata for DISC samples is:

- Investigative ID: ORI of investigative agency (10 characters). This ORI is the agency's ORI that the investigative agency monitors 24 hours a day for NCIC Hit Confirmation.

- Investigative Case Tracking ID: (32 characters) Investigative agency's reference/case ID
- Investigative phone number: (14 characters format (xxx) xxx-xxxx) Point of Contact of case
- Statute of Limitations: (MM-DD-YYYY): Information must be obtained from investigator or prosecutor's office
- Offense Description: (64 characters): Description of type of crime that yielded type of evidence
- Extradition Information: (255 characters): Investigating agency must be willing to extradite; minimum information is "yes"
- Investigating Agency Contact Information: (255 characters): Any additional contact information or contacts such as contacts to investigative agency's 24 hour Wants and Warrants desk or other case specific information

4.5.4 Eligibility of DISC samples must be re-examined every year. Contact shall be made by the casework LDIS administrator (or any qualified DNA Examiner or any person with administrative rights who has been trained to use the CODIS software or anyone with access to STACS) with the investigative agency to confirm the case is still active and the profile continues to meet DISC eligibility requirements.

4.5.5 DISC samples will be periodically checked for hits to SDIS/NDIS samples by the casework LDIS administrator (or any person with administrative rights who has been trained to use the CODIS software). If the source ID has changed to 'Yes', these DISC samples will be un-enrolled as these samples are no longer eligible for DISC.

4.6 Review of the Match/Rank Detail Report

4.6.1 The casework LDIS Administrator (or any qualified DNA Examiner or any person with administrative rights who has been trained to use the CODIS software) will review the Match/Rank Detail Reports and supporting case file documentation, if necessary, to determine if confirmation of the match/rank is required if the match/rank is at high stringency.

4.6.1.1 The casework LDIS Administrator (or any qualified DNA Examiner) will review the Match/Rank Detail Reports and supporting case file documentation, if necessary, to determine if confirmation of the match/rank is required if the match/rank is at moderate or low stringency.

4.6.1.2 If the candidate match is determined to be a match and confirmation is required, the casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will record the following information, as appropriate:

- Match ID #
- FBI Specimen ID #
- State Specimen ID #
- Match stringency
- Indices of specimens (e.g., Forensic vs. Forensic, Forensic vs. Convicted Offender)

- Point of contact information for other laboratory (or Federal DNA Database Unit)
- Results of search for previous CODIS matches

4.6.1.3 For CODIS Missing Persons candidate ranks, the casework LDIS Administrator (or any qualified DNA Examiner) will review any calculated ranking information the software may have given to a match (i.e., Joint Pedigree Likelihood Ratio (JPLR) and/or combined Likelihood Ratio (LR)) and supporting case file documentation, if necessary, in an effort to evaluate the association. The following information will be recorded as appropriate:

- Rank ID #
- FBI Specimen ID #
- Pedigree ID#
- Indices of specimens (e.g., UHR vs. Pedigree, UHR vs. Convicted Offender)
- Point of contact information for other laboratory (or Federal DNA Database Unit)
- Results of search for previous CODIS ranks

4.6.2 If the candidate match/rank is determined not to be an association the casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will disposition the match/rank accordingly and no further action is required.

4.6.3 Forensic Sample to Forensic Sample Match

A forensic match occurs when two or more DNA profiles developed from crime scene evidence submitted from forensic laboratories match to one another.

- a. The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will contact the matching forensic laboratory to verify the match and exchange case information, as detailed in the *NDIS Operational Procedures Manual*. This information exchange will be recorded in the case file. The contributing agency will be provided with information regarding the forensic match. If appropriate, the casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will change the source ID box in LDIS.

4.6.4 Forensic Sample to Offender Sample Match

An offender match occurs when a DNA profile obtained from a forensic sample matches to a convicted offender, arrestee, detainee and/or legal sample.

- a. The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will determine if the contributor of the forensic sample is unknown or if the sample was reported as a match to a subject. Additional information may be collected from appropriate FBI databases to determine if the case is solved.

- b. If the candidate forensic sample is from a solved case, the casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will confirm the identity of the subject with the convicted offender laboratory and disposition as a conviction match upon name confirmation.
- c. If the candidate forensic sample is from an unsolved case, the casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will contact the convicted offender laboratory for confirmation of the hit, as detailed in the *NDIS Operational Procedures Manual*. This information will be recorded in the case file.
 - 1. In the case of a non-qualifying offense, the contributor will be provided with written notification which should include the offender's name if provided by the offender laboratory and a point of contact for the offender laboratory.
 - 2. If upon evaluation of a hit, it is determined that a forensic sample is not eligible for database entry, the sample will be removed from the database. The contributor will be provided with written notification which should include the offender's name and information regarding the CODIS ineligibility of the forensic sample.
- d. The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will update the source ID box from 'No' to 'Yes', if appropriate.
- e. For Unsolicited DNA Notifications (UDN) for Rapid hits received by the CODIS message center, the casework LDIS administrator (or any person with administrative rights who has been trained to use the CODIS software) will retrieve these UDN messages and contact the contributor to verify that the contributor has received their copy of the UDN and that the contributor is following up on this investigative lead in a timely fashion.

4.6.5 The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will determine whether or not the match provided investigative information and provide this to the CODIS Unit.

4.6.6 Appropriate forensic and offender matches will be verified and recorded by an appropriately qualified DNA Examiner. Written notification containing the match information will be provided to the contributing agency.

4.6.6.1 For forensic mixtures and forensic targeted specimens (as appropriate), matches to single source samples (with the exception of conviction matches) will be verified using STRmix™. A match will be declared if the likelihood ratio (LR) is at least approximately one in the size of the NDIS database for an NDIS match and at least approximately one in the size of the SDIS database for an SDIS match.

- a. If a new STRmix™ deconvolution is needed in order to evaluate a match, this deconvolution must be technically reviewed by an appropriately qualified DNA examiner prior to issuing the written notification to the contributing agency.
- b. If an LR from Previous run from STRmix™ is needed in order to evaluate a match, only an administrative review by the Unit Chief (or any qualified DNA examiner) is required prior to issuing the written notification to the contributing agency.

4.6.7 Missing Persons Matches/Associations

4.6.7.1 A Missing Persons association occurs when a rank is evaluated by the casework LDIS Administrator (or any qualified DNA Examiner) and determined to potentially be an association between a UHR and relatives of that Missing Person. Ranks are evaluated on the following information, if applicable:

- Joint Probability Likelihood Ratio/Combined Likelihood Ratio
 - Lineage Markers
 - Metadata
- a. The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will verify that the corresponding metadata between the UHR and family reference sample(s) is consistent and contact the other laboratory to exchange case information, if necessary, as detailed in the *NDIS Operational Procedures*. This information will be recorded in the case file.
 - b. Written notification will be provided to the contributing agency to include supporting statistical information (e.g., kinship index), as appropriate. Any notification containing statistical information must be technically reviewed by an appropriately qualified DNA Examiner.

4.6.7.2 Missing Persons/UHR matches to Offender Samples occur when a DNA profile obtained from a Missing Person or UHR sample matches to a convicted offender sample.

- a. The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will contact the convicted offender laboratory for confirmation of the hit, as detailed in the *NDIS Operational Procedures*. This information will be recorded in the case file.
- b. Upon receiving confirmation from the convicted offender laboratory, written notification will be provided to the contributing agency to include supporting statistical information (e.g., likelihood ratio as appropriate), when appropriate. Any notification containing statistical information must be technically reviewed by an appropriately qualified DNA Examiner.

4.6.7.3 A lineage marker only association occurs when an association is made between a UHR containing no or very limited autosomal STR data and a pedigree which includes mtDNA or Y-chromosome data.

- a. The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will verify that the corresponding metadata between the UHR and family reference sample(s) is consistent and contact the other laboratory to exchange case information, if necessary, as detailed in the *NDIS Operational Procedures*. This information will be recorded in the case file.
- b. Written notification will be provided to the contributing agency to include supporting statistical information, when appropriate. Any notification containing statistical information must be technically reviewed by an appropriately qualified DNA Examiner.

4.6.8 Written Notification of a Match or Positive Association

4.6.8.1 All written notifications will be administratively reviewed and technically reviewed, as appropriate, by the Unit Chief (or any qualified DNA Examiner) in accordance with the appropriate Laboratory Operations Manual (LOM) practice(s). This review will also verify the following information:

- Administrative information, if applicable (including specimen/sample, investigator, offender).
- Technical information related to missing persons associations, if applicable.
- Profile and specimen ID submitted for entry into CODIS are consistent with the profile and specimen ID on the CODIS Match Detail Reports, if applicable.

4.6.8.2 The administrative and, if appropriate, technical reviewer will record the completion of their review. This record will consist of the signature of the administrative and, if appropriate, technical reviewer and the date of the review. Records regarding the hit (e.g., Match Detail Report, a copy of the report) will be maintained in accordance with the appropriate LOM practices.

4.6.8.3 Written notifications will be issued in accordance with the appropriate LOM practices.

4.7 CODIS Search Requests

4.7.1 The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will update the NDIS batch target file and search against the forensic and missing persons/UHR samples monthly. The date the batch target file is updated and searched as well as the initials of the individual performing the update and search will be recorded.

4.7.2 One-time search requests of LDIS/SDIS for forensic DNA profiles from other NDIS participating laboratories will be individually evaluated by the casework LDIS Administrator, casework LDIS Alternate Administrator, FBI State Administrator or FBI State Alternate Administrator prior to initiating a manual keyboard search. One-time search requests of other laboratories must be approved and requested by the casework LDIS Administrator, casework LDIS Alternate Administrator, FBI State Administrator, or FBI State Alternate Administrator.

4.7.3 A candidate match found with a target profile will be reviewed by the casework LDIS Administrator (or any qualified DNA Examiner) for moderate or low stringency hits and by the casework LDIS Administrator (or any qualified DNA Examiner or any person with administrative rights who has been trained to use the CODIS software) for high stringency hits and written notification will be provided if necessary.

4.7.4 No “victim or suspect” searches will be conducted of the FBI LDIS/SDIS data.

4.8 NDIS Manual Keyboard Searches by NDIS Custodian

4.8.1 A manual keyboard search may be requested if a forensic DNA profile from a serious violent crime (1) must be searched before the routine upload of DNA data due to exigent circumstances or (2) does not meet the required minimum number of CODIS Core Loci for uploading the DNA profile to NDIS but does contain at least 7 of the original 13 CODIS Core Loci and satisfies a statistical threshold for match rarity based upon the search criteria by locus of one in ten million. A manual keyboard search shall not be used in place of the routine upload and search.

4.8.2 The qualified DNA Examiner requesting a keyboard search will ensure the request complies with the current NDIS Procedures.

4.8.2.1 The DNA profile to be keyboard searched must be interpreted by a qualified DNA Examiner. The keyboard search request records and supporting data for the profile and the associated controls must be technically reviewed by a second qualified DNA Examiner and the profile must be eligible for retention in the database (i.e., LDIS, SDIS, or NDIS). The profile to be searched and record of the interpreting Examiner and Technical Reviewer must be recorded in the case file.

4.8.3 The casework LDIS Administrator, casework LDIS Alternate Administrator, or a DNA Examiner with CODIS software training will search the profile requested for keyboard search in LDIS.

4.8.4 The keyboard search request records, including record of the interpreting Examiner and Technical Reviewer, will be forwarded to the FBI State Administrator or FBI State Alternate Administrator who will formally request, in writing, the NDIS search.

4.8.5 A candidate match from a manual keyboard search will be reviewed by the casework LDIS Administrator (or any qualified DNA Examiner) for a moderate stringency hit and by the casework LDIS Administrator (or any qualified DNA Examiner or any person with

administrative rights who has been trained to use the CODIS software) for a high stringency hit and written notification will be provided if necessary.

4.9 Removal of a Sample from CODIS

4.9.1 Deletion of a DNA profile in response to a court order that is specific to removing a forensic unknown DNA profile from CODIS is referred to as an expungement.

4.9.1.1 The casework LDIS Administrator, casework LDIS Alternate Administrator or Missing Persons Program Manager will review the court order to ensure it is complete.

4.9.2 Deletion of a DNA sample from CODIS upon verification of the fact that the DNA record is not eligible or suitable for inclusion in CODIS is known as an administrative removal. Administrative removal may be warranted if:

- The contributing agency notifies the laboratory in writing that removal is warranted.
- Additional information indicates the sample is not appropriate for CODIS and/or does not meet the requirements set forth in the *NDIS Operational Procedures Manual*.
- An identification has been made for a Missing Person/UHR.

4.9.3 The casework LDIS Administrator (or any person with administrative rights who has been trained to use the CODIS software) will delete the DNA profile using the CODIS software and record this action electronically (see *NDIS Operational Procedures Manual*). The LDIS specimen deletion report will also be maintained electronically.

4.10 System Operations - Backup Procedures

4.10.1 The LDIS backup will be conducted by an FBI employee or approved contractor.

4.10.2 The backup will include all relevant drives and the registry from the LDIS server.

4.10.3 Tapes are stored in safes at the FBI Laboratory and at an off-site location.

4.10.4 In the event of an emergency and/or catastrophic loss, the backup tapes may be used to restore the LDIS.

4.11 Security

4.11.1 Only authorized personnel will have physical access to CODIS servers and terminals.

4.11.2 All CODIS users are responsible for protecting the security of the software as a user.

4.11.3 When a user is finished with a CODIS work session, the user will log out of CODIS. If the user has not completed their CODIS work session but needs to move to an area in which

they can no longer visually observe the CODIS server/terminal, they must lock the screen or log out of CODIS.

5 Standards and Controls

Not applicable.

6 Sampling or Sample Selection

Not applicable.

7 Calculations

Not applicable.

8 Measurement Uncertainty

Not applicable.

9 Limitations

9.1 A legal expungement cannot be performed without a court order signed by a judge.

9.2 All profiles entered into the Forensic Index maintained by the DNA Casework Unit (DCU) or Biometrics Analysis Unit (BAU) will meet the criteria described in “*A Guide to Determining What is Allowable in the Forensic Index at NDIS.*”

10 Safety

Not applicable.

11 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Operations Manual (LOM)

DNA Procedures Manual

National DNA Index System (NDIS) Operational Procedures Manual

Guide to Determining What is Allowable in the Forensic Index at NDIS

Federal Bureau of Investigation, Quality Assurance Standards for Forensic DNA Testing Laboratories, latest version.

Case Management Users Manual, RoboTech Sciences, latest version.

Rev. #	Issue Date	History
12	10/26/18	<p>Changed DCU Examiner to DNA Examiner throughout. Added qualified to DNA Examiner throughout. Removed CODIS from State Administrator titles throughout. 3 Changed to version 8.0 or higher. 4.1.2 Added additional example. 4.2.1 Relocated from 4.2.2.2. 4.2.3.1 Relocated and added info describing specimen categories. 4.2.3.4 Put requirements for specimens in table. 4.2.4 to 4.2.7 Added additional info for missing person case samples. 4.5 Added DISC information. 4.6.4 Added Rapid hit info in e. 4.6.7.1 Specified for legacy cases that are not assigned a lab number in FA. 4.6.9.3 Revised to apply to lineage marker associations. 4.10.2 Removed specific server designation</p>
13	04/26/19	<p>4.2.3.1 Listed the 13 CODIS core loci 4.2.3.2 Added forensic targeted 4.2.3.5 Added forensic targeted and marking partial loci 4.6.6.1 Added guidance for using STRMix 4.6.8 Relocated written notification info from 4.6.7 and added header.</p>

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 04/24/2019

DCU Chief

Date: 04/24/2019

Acting BAU Chief

Date: 04/24/2019

QA Approval

Quality Manager

Date: 04/24/2019

Procedures for Amplification of Nuclear DNA

1 Scope

These procedures apply to DNA personnel who prepare plates for the amplification of nuclear DNA (nDNA) from evidence and reference samples and DNA personnel that perform the associated quality control procedures. The DNA Casework Unit (DCU) and Biometrics Analysis Unit (BAU) use Sample Tracking and Control Software (STACS) and robotic workstations to automate the set-up of the amplification (aka amp) plates.

2 Equipment/Materials/Reagents

Equipment/Materials

- Tecan robot model 'Freedom EVO'
 - Tecan EvoWare software, version 2.3 or higher
- STACS, version 3.2.920 or later
- Thermal Cycler, GeneAmp® PCR System 9700
- General laboratory supplies (e.g., pipettes, tubes)
- 96-well plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear and foil plate seals
- Thermal Microplate Sealer

Reagents

- AmpF/STR® PCR Amplification Kit(s) (Applied Biosystems)
 - Identifiler® Plus
 - Yfiler™
 - 9947A, 10 ng/μL
- GlobalFiler™ PCR Amplification Kit (Applied Biosystems)
- 007 or 9947A sample, quantified and diluted as necessary (Applied Biosystems)
- Buffer, Low TE (aka TEKnova DNA Suspension Buffer) (Fisher Scientific or equivalent)
- 3% bleach (household or equivalent)
- 10% bleach (Daigger or equivalent)
- Isopropyl alcohol, 70%
- Water (reagent grade or equivalent)
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution (Liquinox™ or equivalent)

3 Standards and Controls

A positive amplification control and negative amplification control (also referred to as the amplification blank) must be processed in parallel with each set or batch of evidentiary samples

subjected to polymerase chain reaction (PCR) amplification. 9947A DNA is the positive control for the Identifiler® Plus kit and 007 DNA is the positive control for the GlobalFiler™ and Yfiler™ kits. The maximum volume of reagent grade water that can be accommodated by the PCR volume (i.e., 10 µL) is the negative control. The positive and negative amplification controls must be amplified concurrently (i.e., in the same instrument and with the same primers) with the forensic samples to which they will be associated.

At least one reagent blank (RB) from an extraction batch must be amplified using the same primers, same instrument model, and same concentration conditions as required by the sample(s) in the extraction batch containing the least amount of DNA. For extraction batches with multiple RBs, at least the RB that demonstrates the greatest signal, if any, must be amplified. An amplification test kit may not be utilized if no RB(s) associated with the extraction batch or sample being amplified remains.

Refer to the appropriate interpretation procedure of the *DNA Procedures Manual* for interpretation guidelines of these control samples.

4 Procedures

Refer to the DNA Introduction Procedure (i.e., DNA QA 600) and follow applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water will be used. The purified water, available via faucets (typically labeled DE) at the laboratory sinks, is used for Tecan operation and is also called Tecan system liquid.

Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

4.1 Preparation of the Tecan Robotic Workstation

If necessary, turn on the Tecan, which will undergo an initialization routine. Log on to the Tecan computer, launch and log on to the current Tecan software.

4.1.1	<p>Ensure the Tecan is prepared to run:</p> <p>Prior to daily use:</p> <ul style="list-style-type: none"> • Make ~100 mL of 3% bleach to replace bleach in front trough • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script <p>Prior to each run:</p> <ul style="list-style-type: none"> • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. <i>If replacing carboy, rerun daily start up script. When a carboy is refilled, it should be allowed to de-gas overnight</i> 	
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	<p><i>before use.</i></p> <ul style="list-style-type: none"> • Check volume of waste container and empty if needed <p>As needed:</p> <ul style="list-style-type: none"> • Clean barcode scanners with a lint-free cloth. 	
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The daily start up script prompt “Check syringes and tips,” refers to checking that the tubing and syringes (plunger lock screws) are tight and not introducing air bubbles, and that the tips are tight, free of clogs, and not leaking.

RoboScrub cleaning should be performed weekly, generally at the end of the workday:

- Make ~3.5 L of diluted Liquinox solution (see instructions on the label of the bottle for preparation)
- ~3.5 L purified water in a separate container is needed
- Run the RoboScrub Clean script, and follow the prompt

4.2 Preparing Sample Racks/Creating a Scan File Import

Ensure all DNA extracts and reagent blanks (aka DNA sample tubes) are in Tecan compatible tubes and appropriately barcoded.

4.2.1	<p>Place tubes into Tecan samples rack(s) as described below and load the sample racks onto the Tecan.</p> <ul style="list-style-type: none"> • A ladder place holder tube (i.e., uncapped empty tube with a unique “BL” barcode) is in position 1 of sample rack 1. • DNA sample tubes start in position 2 of sample rack 1 and continue on to additional racks as needed. <ul style="list-style-type: none"> ○ When more than 4 sample racks are used, a ladder place holder tube must be added between the first and last samples. For a full 96-well amplification plate, this is generally position 13 of rack 3. • A tube of positive control DNA (with an appropriate [i.e., FC, MC] barcode) is in the sample position immediately after the last DNA sample tube. <ul style="list-style-type: none"> ○ 007 ("MC...") is used for GlobalFiler™ and Yfiler™ ○ 9947A ("FC...") is used for Identifiler® Plus • A new barcoded amplification blank ("AMPBLANK") tube is in the sample position immediately after the positive control DNA. • A ladder place holder tube is immediately after the amplification blank tube. • Any rack position(s) unfilled by a tube as described above must contain empty tubes with unique “BL” barcodes. 	
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“BL” barcode tubes may be reused; however, each “BL” barcode on the Tecan must be unique.

4.2.2	Use the current appropriate script to scan the sample racks and generate a .csv scan file. Import the scan file into STACS.	
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4.3 Master Mix Preparation

This step may be performed any time prior to loading the master mix on the Tecan robot.

4.3.1	Create master mix based on the following volumes. Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin. Ensure the appropriate barcodes are entered into STACS. Generate Amp File in STACS.	
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Amplification Master Mix Components

GlobalFiler™ (GF)		Yfiler™ (Y)		Identifiler® Plus (ID)	
	µL per well*		µL per well*		µL per well*
GF Master Mix	7.5	Y Reaction Mix	9.2	ID Master Mix	10
GF Primer Set	2.5	Y Primer Set	5	ID Primer Set	5
Low TE Buffer	5	AmpliTaq Gold®	0.8		

*Number of wells = number of samples + ~11-12 (for controls, ladders, and overage)

4.4 Preparing the Tecan Deck

The below steps may be performed in any order prior to running the Tecan robot.

Positions of racks may vary between instruments. The robotic script will direct the placement.

4.4.1	Water Rack: <ul style="list-style-type: none"> Prior to each use, replace the ~200 mL reagent grade water in the center trough. 	
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Maximum penetration depth for the robot tips is 0.75 inches from the bottom of the trough (threshold line is on the trough). Observe the water level during processing and replenish if necessary.

4.4.2	Bleach Rack: <ul style="list-style-type: none"> Ensure the 3% bleach solution in the front trough was replaced prior to daily use. 	
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4.4.3	Plate Rack: <ul style="list-style-type: none"> Place a 96-well plate into a base. Place into the back position of the plate rack. Ensure an amplification batch barcode label is on the right side of the base or the plate. 	
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	<ul style="list-style-type: none"> Label the right side of a 96-well 2.0 mL deep well plate with a working plate barcode and place it into the center position of the plate rack. 	
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4.4.4	Master Mix Rack: <ul style="list-style-type: none"> Place the two tubes (with “C1” barcodes) containing equal volumes of master mix in positions 3 and 4. Place empty tubes (with unique “BL” barcodes) in positions 1 and 2, and 5 through 16. 	
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4.5 Tecan Amplification Plate Set-up

4.5.1	Run the current version of FBI AMPSTR then run appropriate Amp Script. Follow the prompts to ensure the appropriate racks are on the deck as required.	
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Based on each sample’s quantification (quant) result and any adjustments made by an examiner, the Tecan will create the necessary dilutions using reagent grade water. The dilution plate can take more than 30 minutes to generate.

4.5.2	Follow the script prompts for the dilution plate. The script will include prompts to perform the following tasks. <i>If no dilutions were prepared, the dilution plate does not need to be manipulated while following the script prompts.</i>	
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4.5.2.1	Seal the dilution plate with a foil cover.	
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The seal may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are well-sealed.

4.5.2.2	Invert the dilution plate several times, attempting to shake the liquid off the bottom of the plate.	
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4.5.2.3	Firmly seat the dilution plate on the te-shake and follow computer prompts to turn on the plate shaker.	
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The plate will shake for ~1.5 minutes.

4.5.2.4	Remove the dilution plate from the te-shake and invert several additional times, attempting to shake the liquid off the bottom of the plate. Centrifuge the dilution plate for ~1.5 minutes at ~2000 rpm. Remove the dilution plate from the centrifuge and carefully remove the foil seal. Return the dilution plate to its original deck position in the correct orientation .	
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Ensure that the plate is properly positioned **BEFORE** selecting “OK” to continue with the script.

The Tecan will prepare the amp plate so that each well contains 15 µL of master mix and 10 µL of DNA template (i.e., neat sample, sample plus water, diluted sample) or 10 µL of the appropriate control. See the calculations and limitations section for more information.

4.5.3	Upon completion of the Tecan run, seal the amplification plate with a clear seal.	
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The seal may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are well-sealed.

4.5.4	Quick spin (generally ~2,000 rpm for 5 seconds).	
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Sample tubes should be removed from the Tecan deck and capped prior to proceeding to the Amplification (Amp) room.

4.5.5	Ensure the amp batch barcode is on a side of the amplification plate and proceed to the Amp room.	
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Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

4.6 PCR Amplification

4.6.1	Place the amp plate in an appropriate thermal cycler. Place an optical compression pad, gold side up, onto the top of the sealed plate and close the lid by pressing the lever down completely.	
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4.6.2	Select the programmed amplification method to run (e.g., “GlobalFiler”).	
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Each thermal cycler is programmed for one of the following methods.

GlobalFiler™

HOLD 95°C 1 minute
CYCLE 94°C 10 seconds
59°C 90 seconds
Repeat for 28 total cycles
HOLD 60°C 10 minutes
HOLD 4°C ∞

Identifiler® Plus

HOLD 95°C 11 minutes
CYCLE 94°C 20 seconds
59°C 3 minutes
Repeat for 27 total cycles
HOLD 60°C 10 minutes
HOLD 25°C ∞

Yfiler™

HOLD 95°C 11 minutes
CYCLE 94°C 1 minute
61°C 1 minute
72°C 1 minute
Repeat for 28 total cycles
HOLD 60°C 80 minutes
HOLD 25°C ∞

4.6.3	Follow the prompts to start the method. <ul style="list-style-type: none"> For GlobalFiler: Ensure the reaction volume is 25 µL and the ramp speed is MAX. For Identifiler Plus and Yfiler: Ensure the reaction volume is 25 µL and the ramp speed is 9600. 	
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Upon completion of the PCR amplification, the plate should be stored refrigerated until needed for capillary electrophoresis (CE).

5 Calculations

The volume of DNA extract used in the amplification is calculated using the quant results of the sample, the maximum volume of sample allowed in the amplification reaction, and the target DNA amounts. An examiner may adjust the volume of sample amplified, as needed. An examiner can override or adjust the dilutions that the Tecan will make within STACS.

The following is the basis for determining the volume of sample to use for amplification:

$$C_1V_1 = C_2V_2 \rightarrow V_1 = (C_2V_2)/C_1$$

Where C_1 = Quant result (ng/µL)

V_1 = Volume of sample to add to amplification reaction (µL)

C_2 = Target Concentration (i.e., 0.1 ng/µl)

V_2 = Maximum input volume (i.e., 10 µL)

Example inputs:

DNA sample quant result (ng/µl)	Volumes to add to amplification for target concentration of 1 ng per 10 µL
<0.1	10 µl sample
0.1	10 µl sample
0.25	4 µl sample + 6 µl water
0.5	2 µl sample + 8 µl water

6 Sampling or Sample Selection

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 Successful amplification is dependent upon the quantity or quality of DNA in the sample.

8.2 Target DNA amounts are determined during validation and set in the software. The software uses the quantitation results and the target DNA amount to determine the volume of input of DNA or the default dilution calculation. Generally, 1 ng of DNA is targeted but the input volumes may be adjusted by an examiner such that more or less DNA sample is used. For samples that quant at less than 0.1 ng/μL, the default is the maximum input (i.e., 10 μL).

8.2.1 The Tecan is not maintained to pipette less than 2 μL. As a result, with a target of 1 ng/10μL, the default is to make a dilution for any sample with a quant value >0.5 ng/μL. An examiner should adjust the amplification setup sheet information, as appropriate, to prevent the Tecan from using more sample than necessary to make a dilution while ensuring an appropriate dilution volume is queued.

8.2.1.1 When adjusting the amplification setup information, generally the minimum volume accepted for a dilution is 40 μL total (i.e., dilution buffer plus sample) and the accepted maximum volume of dilution buffer is 1200 μL.

8.2.2 The Tecan has a maximum dilution that can be made. Generally, any sample with a quant value >300 ng/μL will need to be manually diluted and requanted prior to amplification.

8.3 Replicate amplifications may assist with statistical interpretation using STRMix. Replicate amplifications are not required to be at the same input concentration.

8.4 At least one reagent blank from an extraction batch must be amplified using the same primers (i.e., amp kit), same instrument model, and same concentration conditions as the sample(s) containing the least amount of DNA. An additional amplification kit may not be utilized if no reagent blank(s) associated with the extraction batch or sample being amplified remains.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Avoid reaching into the Tecan robot while it is running as personal injury could result from moving robot accessories.

10 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. *GeneAmp® PCR System 9700 User's Manual Set*. 1997.

Applied Biosystems. *GlobalFiler™ PCR Amplification Kit User's Guide*, P/N 4477604 Rev E, 2016.

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Rev. #	Issue Date	History
11	05/25/16	Complete revision for simplification of procedure. Changed from nDNAU to DCU throughout. Changed from nDNAU LIMS to STACS throughout and made necessary adjustments for STACS. Moved QC procedures to Appendix and simplified.
12	12/30/16	Updated to add GlobalFiler and remove MiniFiler. Incorporated BAU for when Huntsville lab is authorized to perform procedure on casework.
13	02/28/18	1 Adjusted scope. 4.4.3 Added allowance for different placement of the barcode based on the Tecan plate holder. Appendix A, 2 Critical Reagents, section C made triplicate for consistency.

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 02/27/2018

DCU Chief

Date: 02/27/2018

BAU Chief

Date: 02/27/2018

DSU Chief

Date: 02/27/2018

QA Approval

Quality Manager

Date: 02/27/2018

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency of performance verifications and additional requirements.

A. Performance Verification (PV) of the Tecan Robotic Workstation

Refer to the nuclear DNA quantification procedure (i.e., nDNA 226) for instructions for the PV of the Tecan Robotic Workstation.

B. Performance Verification of the 9700 Thermal Cycler

1. **Cleaning** - Refer to the instructions for cleaning the sample wells and cleaning the sample block cover described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set.
2. **Temperature Verification Test** - This procedure verifies that the thermal cycler remains within the temperature accuracy specification. Refer to the instructions for running the Temperature Verification Test described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set. This test procedure requires the use of a Temperature Verification System.
3. **Temperature Non-uniformity Test** - This procedure verifies the temperature uniformity of the sample wells in the thermal cycler. Refer to the instructions for running the Temperature Non-uniformity Test described in the maintenance section of the GeneAmp® PCR System 9700 User's Manual Set. This test procedure requires the use of a Temperature Verification System.
4. **Rate Test and Cycle Test** - These procedures verify the integrity of the cooling and heating system of a thermal cycler. Refer to the instructions for running system performance diagnostics described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

Qualification of GlobalFiler™, AmpF/STR® Identifiler® Plus, and AmpF/STR® Yfiler™ Amplification Kits

A. Normalization of Positive Control DNA

1. The positive control DNA (e.g., 007, 9947A) from each new lot of an amplification kit will be quantitated. Generally, in triplicate and the quant values averaged.
2. If necessary (i.e., when the average concentration is greater than ~ 0.10 ng/ μ L), a portion of the positive control DNA will be adjusted with an appropriate diluent (e.g., TE⁻⁴ or reagent grade water) to a concentration of ~ 0.10 ng/ μ L.
3. The positive control DNA, at the ~ 0.10 ng/ μ L concentration, will be used for the assessment of the sensitivity of detection and kit performance.

B. Sensitivity of Detection

1. Using an amplification kit from the new lot, amplify the normalized positive control DNA (generally in triplicate), a negative amplification control, and a diluent control (if applicable). Run the samples on the CE instrument at current conditions.
2. Evaluate the CE data and compare the data generated for the positive control to the expected sensitivity results. Sensitivity expectations for a given amplification kit are based on the evaluation of multiple lots of the kit and positive control during validation.
3. The sensitivity of the new lot will be accepted if the average allelic peak heights for all successful injections of the positive control DNA are generally consistent with sensitivity expectations for the given amplification kit.
4. If the amplification kit does not meet sensitivity expectations, the assessment will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

C. Positive Control and Kit Performance

Once a new lot of an amplification kit has demonstrated an acceptable sensitivity of detection, a batch of the normalized positive control DNA will be prepared for use in casework analysis.

1. Using an amplification kit from the new lot, amplify, generally in triplicate, a sample from this batch of normalized positive control DNA, a negative amplification control, and a diluent control (if applicable). For a new lot of Yfiler™ Amplification Kit, the 9947A control DNA (10 ng/μL) is run as an additional negative control sample. Run the samples on the capillary electrophoresis instrument at current conditions using the new lot of allelic ladder.
2. Evaluate the CE data. Only 1 replicate of each sample is needed for the evaluation.
3. The amplification kit lot and the positive control DNA preparation will be approved for casework analysis if:
 - a. all allelic ladder peaks are present for at least one injection of the allelic ladder
 - b. correct and interpretable typing results are obtained for the positive control DNA
 - c. no allelic peaks, other than those attributable to the amplified positive control DNA, are detected
 - d. no allelic peaks are detected in the negative control and diluent control (if applicable)
 - e. the allelic peak heights of the positive control DNA are generally consistent with sensitivity expectations for the given amplification kit.
4. If the injection set does not meet the above listed criteria, the assessment will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.
5. The approved neat or adjusted preparation of the positive control DNA will be stored in 1.5 mL screw cap tubes affixed with appropriate positive control barcodes for usage.

DNA

Procedure for the Capillary Electrophoresis of Nuclear DNA

1 Scope

These procedures describe the separation by capillary electrophoresis (CE) of amplified nuclear DNA (nDNA) products from evidence and reference samples. The DNA Casework Unit (DCU) and Biometrics Analysis Unit (BAU) use Sample Tracking and Control Software (STACS) and robotic workstations to automate the set-up of the CE plates.

2 Equipment/Materials/Reagents

Equipment/Materials

- STACS, version 3.2.920 or higher
- Agilent Technologies “Bravo” Liquid Handler
 - VWorks Software, version 11.0.0.874 or higher
- Liquid handler tips, Agilent Technologies or equivalent
- Thermal Cycler, GeneAmp® PCR System 9700
- 3130xl Genetic Analyzer, Applied Biosystems
 - Data Collection Software, version 3.0 or higher
 - 16-Capillary (16-cap) Array, 36 cm
- 3500xL Genetic Analyzer, Applied Biosystems
 - Data Collection Software, version 3.1 or higher
 - 24-Capillary (24-cap) Array, 36 cm
- GeneMapperID-X, version 1.4 or higher
- General laboratory supplies (e.g., pipettes, tubes)
- 96-well plate, Applied Biosystems MicroAmp® optical or equivalent
- Plate septa, Applied Biosystems or equivalent
- Plate base and retainer, Applied Biosystems or equivalent

Reagents

- Hi-Di™ formamide, Applied Biosystems or equivalent
- GeneScan™ -500 LIZ™ internal size standard, Applied Biosystems
- GeneScan™ -600 LIZ™ Version 2.0 internal size standard, Applied Biosystems
- Ladder from the appropriate Amplification Kit, Applied Biosystems
 - Identifiler® Plus (ID+) Ladder
 - Yfiler™ (Y) Ladder
 - Globalfiler® (GF) Ladder

- 3130xl Instrument Reagents:
 - 1X Genetic analyzer buffer with EDTA, Applied Biosystems or equivalent
 - Performance Optimized Polymer 4 (POP-4™), Applied Biosystems
- 3500xL Instrument Reagents:
 - Anode Buffer Container (ABC) 3500 series, Applied Biosystems
 - Cathode Buffer Container (CBC) 3500 series, Applied Biosystems
 - Performance Optimized Polymer 4 (POP-4) Pouch 3500 series, Applied Biosystems
 - Conditioning Reagent, Applied Biosystems
- DS-33 Matrix Standard Kit (Dye Set G5)[For ID+ and Y], Applied Biosystems
- DS-36 Matrix Standard Kit (Dye set J6) [For GF], Applied Biosystems
- Isopropyl alcohol, 70%
- Water, reagent grade or equivalent
- Purified Water or equivalent, available at laboratory sinks

3 Standards and Controls

The positive and negative amplification controls must be subjected to CE analysis in parallel with the associated set or batch of evidentiary samples. The positive must be included in any reparation of samples for CE.

The amplified reagent blank (RB) from an extraction batch must be subjected to CE analysis using the same instrument model, same injection conditions, and most sensitive volume conditions as required by the sample(s) in the associated extraction batch.

Refer to the appropriate nuclear DNA interpretation procedure of the *DNA Procedures Manual* for interpretation of these controls.

4 Procedures

Refer to DNA Introduction Procedure (i.e., DNA QA 600) and follow applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., CE reservoirs), reagent grade, or equivalent, water will be used. The purified water, available via faucets (typically labeled DE) at the laboratory sinks, may be used for rinsing instrument components (e.g., Bravo reagent trough).

Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

4.1 Formamide: Internal Size Standard (ISS) Mixture (aka LIZ Formamide)

If needed, prepare the LIZ Formamide mix in the ratio listed below. Record the preparation in STACS. The LIZ Formamide mix may be stored refrigerated for up to one week.

	GlobalFiler on 3500xL	Identifiler Plus or Yfiler on 3130xl
Size Standard	GS-600 LIZv2	GS-500 LIZ
Hi-Di formamide: LIZ ratio	24:1	99:1

Example Calculations:

GS-600 LIZ Formamide (10 mL): 9.6 mL Hi-Di formamide + 400 µL GS-600 LIZv2

GS-500 LIZ Formamide (20 mL): 19.8 mL Hi-Di formamide + 200 µL GS-500 LIZ

4.2 CE Daughter Plate Preparation

If an Agilent Bravo Liquid Handler is unavailable, the CE daughter plate may be prepared manually.

For manual daughter plate prep:

4.2.1	<p>For Identifiler Plus or Yfiler on 3130xl:</p> <ul style="list-style-type: none"> • Add 24 µL GS-500 LIZ Formamide to each well (or set of 16 wells) in the CE daughter plate, • Add 1 µL of amplified product (excluding the ladder wells) from the amplification plate to the corresponding wells on the CE daughter plate. <p>For GlobalFiler on 3500xL:</p> <ul style="list-style-type: none"> • Add 10 µL GS-600 LIZ Formamide to each well (or set of 24 wells) in the CE daughter plate • Add 1 µL of amplified product (excluding the ladder wells) from the amplification plate to the corresponding wells on the CE daughter plate. <p><i>NOTE: Each set of wells for one injection (i.e., 16 wells on 3130xl, 24 wells on 3500xL) must be filled with LIZ Formamide even if not all wells in that set will be receiving samples.</i></p> <p>Proceed to adding appropriate Ladder.</p>	
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When using the Agilent Bravo Liquid Handler, the work deck must be decontaminated with appropriate cleaner each workday before use and if it becomes visibly soiled. *Bleach should not be used.*

For robotic daughter plate prep:

4.2.2	<p>Ensure the following items are provisioned to the robot and that the appropriate barcodes are entered as required by STACS (see Figure 1):</p> <ul style="list-style-type: none"> • Position 1: A box of Agilent disposable tips. A full box or a half consumed box of tips may be used. • Position 2: The amplification plate, unsealed and in a plate holder. • Position 4: A tip box for used tips. This box must be empty or half empty. An empty box may only be used for 2 plates. • Position 5: A new 96-well plate with a CE daughter plate barcode on the right side. • Position 9: A reagent trough with wells containing a sufficient volume of the appropriate LIZ Formamide. <ul style="list-style-type: none"> - For full plate, enough to cover every well of the reagent trough. - For partial plate on a 3130xl, enough to cover each set of 16 wells (2 columns). - For partial plate on a 3500xL, enough to cover each set of 24 wells (3 columns). <p><i>NOTE: Each set of wells for one injection (i.e., 16 wells on 3130xl, 24 wells on 3500xL) must be filled with LIZ Formamide even if not all wells in that set will be receiving samples.</i></p>	
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Ensure the robot is powered on, logon to the robot's computer, and launch and logon to the VWorks Software.

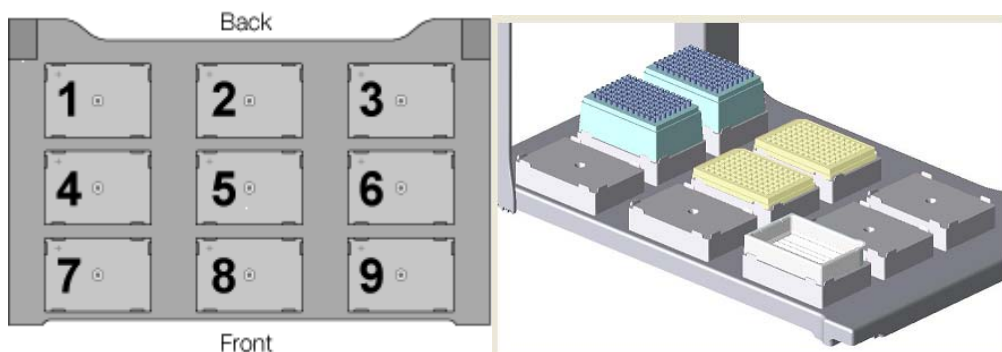


Figure 1 – Agilent Bravo Liquid Handler Deck Positions

4.2.2.1	<p>Initiate the appropriate daughter plate protocol and follow the prompts. Ensure tip positioning for the new and used tip boxes is selected correctly.</p>	
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CAUTION: The instrument is equipped with a safety light curtain. If the operator reaches in during a run, the pipette head motors are disabled and the operation stops.

4.2.2.2	When the run is complete, ensure all appropriate wells contain LIZ Formamide.	
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For the 3130xl: Each set of 16 wells (2 columns of 8) that contain any sample(s), must be filled with liquid to prevent introducing bubbles into the capillaries on the CE instrument.

For the 3500xL: Each set of 24 wells (3 columns of 8) that contain any sample(s), must be filled with liquid to prevent introducing bubbles into the capillaries on the CE instrument.

4.2.3	Add 1 µL of the appropriate allelic ladder to the designated CE daughter plate well(s).	
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4.2.4	Seal the CE daughter plate with septa. 3500xL plates require a full septa. Vortex and quick spin the CE daughter plate.	
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4.2.5	Denature the CE daughter plate in a thermal cycler by using the appropriate method (i.e., Denature).	
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Leave the lid of the thermal cycler unlocked to avoid the septa adhering to it.

Each thermal cycler is programmed with the following method for denaturing:

HOLD 95°C 3 minutes
HOLD 4°C 3 minutes
HOLD 4°C ∞

Ensure the amplification plate is resealed and stored at 4°C until it is appropriate to discard.

The remaining LIZ Formamide may be recovered from the reagent trough and appropriately stored for future use. The reagent trough should be rinsed twice (collect the first rinsate in appropriate waste container), dried, and reused.

4.3 Setting Up the 3130xl

Log on to the computer workstation, ensure the CE is on, and launch the Data Collection software. If the instrument is off, ensure the attached computer is turned on prior to turning on the instrument.

The 1X buffer and reagent grade water in the reservoirs and the POP-4 are generally replaced weekly. If necessary, replenish the reservoirs with 1X buffer and/or reagent grade water, and fill the capillary array with POP-4.

The CE oven temperature may be set to 60°C to allow the CE to warm up and expedite the start of the run.

4.3.1	At any point prior to scheduling the run: Import the plate record created in STACS into the Data Collection software. Verify that all fields are filled in correctly.	
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If necessary, the plate record may be manually created in the Data Collection software.

4.3.2	After denaturation of the CE daughter plate (i.e., anytime during the final 4°C hold), place the plate into a plate base and secure with a plate retainer. Place the CE daughter plate assembly on the CE autosampler with the notched end of the plate assembly facing the right front of the instrument. One or two plates may be loaded on the CE.	
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Ensure the plate retainer seats directly over the septa to avoid damage to the capillary array.

4.3.3	Use the Run Scheduler in the Data Collection software to link the appropriate plate record(s) with the CE daughter plate(s).	
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To confirm that a run has been properly scheduled, the run view can be selected.

4.3.4	Ensure the instrument doors are closed and start the run.	
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Run status, instrument status, event log, raw data, and capillary/array can be monitored during the course of a run.

4.4 Setting Up the 3500xL

Ensure that the oven and all instrument doors are shut and power on the computer, but do not log on. Press the power button on the front of the analyzer to start the instrument. Ensure that the green status light is on before proceeding.

Log onto the workstation and then launch the 3500 Series Data Collection Software application only AFTER the 3500xL Server Monitor has fully initialized.

Check consumable status in the dashboard. Replenish the consumables (POP-4, Anode Buffer Container, Cathode Buffer Container, or Array), if necessary.

Caution: To avoid electrical arcing, all surfaces and containers must be clean and dry.

The CE oven temperature may be set to 60°C to allow the CE to warm up and expedite the start of the run. Ensure the oven is set to 60°C and select the "Start Pre-Heat" button. The preheat function turns off after 2 hours of instrument inactivity.

4.4.1	At any point prior to scheduling the run: Import the plate record created in STACS into the Data Collection software. Verify that all fields are filled in correctly and that the correct Assay has been added to the plate along with the File Name Convention and Results Group	
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If necessary, the plate record may be manually created in the Data Collection software.

4.4.2	After denaturation of the CE daughter plate (i.e., anytime during the final 4°C hold), place the plate into a plate base and secure with a plate retainer. Place the CE daughter plate assembly on the CE autosampler with the notched end of the plate assembly facing the right front of the instrument. One or two plates may be loaded on the CE.	
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Ensure the plate retainer seats directly over the septa to avoid damage to the capillary array.

4.4.3	Click <i>Link Plate for Run</i> or <i>Load Plates for Run</i> in the navigation panel to assign the plate(s) and specify the position of the plate(s) in the autosampler (A and/or B).	
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Confirm that the linked plate(s) are in the correct position of the autosampler. Click *Create Injection List* to review the injection list and/or make any changes or choose *Preview Run* on the left navigation panel before starting the run.

4.4.4	Ensure the instrument doors are closed and start the run.	
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Run status, instrument status, event log, raw data, and capillary/array can be monitored during the course of a run by selecting *Monitor Run* from the navigation panel.

4.5 Data Review

4.5.1	After the CE run, move the data generated by the CE (i.e., .fsa files, .hid files) to the appropriate network folder. Use GMIDX to screen the data for samples that need reinjection or reparation.	
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The entire plate does not need to be run to screen sample data. Sample data may be viewed after each injection is completed.

The plate record may be edited to add injections, as needed, after the completion of the run. When reinjecting samples after the run has ended, a ladder(s) should also be reinjected.

An examiner will review the CE data for compliance with the requirements in the nDNA interpretation procedure of the *DNA Procedures Manual* and queue any samples that need to be rerun (aka reprep).

For samples that require a new CE daughter plate set up (aka reprep), repeat this procedure for at

least the applicable samples. At minimum, the positive amplification control and ladder(s) need to be included and injected. A new plate record is generated for the reprep.

5 Calculations

Not applicable.

6 Sample Selection

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

Not applicable.

9 Safety

9.1 Refer to the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual* for important personal safety information to conducting these procedures.

9.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Procedural Specific Chemical Hazards:

- Formamide is a teratogen. Avoid inhalation, skin contact, or ingestion. Use nitrile gloves when handling. Dispose of unused portions in appropriate hazardous waste containers. Pregnant women must not handle formamide. Any pregnant Biologist should advise a supervisor so that arrangements can be made to have an alternate individual(s) perform all formamide handling procedures.
- Performance Optimized Polymer (POP-4) is caustic. Avoid inhalation, skin contact, or ingestion. Use gloves when handling. Dispose of unused portions in appropriate hazardous waste containers.

10 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. *GeneAmp® PCR System 9700 User's Manual Set*. 1997.

Applied Biosystems. *3130/3130xl Genetic Analyzers Getting Started Guide*. 2004.

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Applied Biosystems. *3500/3500xL Genetic Analyzer User Guide*, Foster City, CA.

Applied Biosystems. *Multi-Capillary DS-33 (Dye Set G5) Matrix Standard Product Insert*. 2004.

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Applied Biosystems. *GlobalFiler® PCR Amplification Kit User Guide*. Applied Biosystems, Foster City, CA.

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ARTEL. *MVS Multichannel Verification System User Guide*. 2006

Rev. #	Issue Date	History
7	05/25/16	Complete revision for simplification of procedure. Changed from nDNAU to DCU throughout. Changed from nDNAU LIMS to STACS throughout and made necessary adjustments for STACS. Moved QC procedures to Appendix.
8	12/30/16	Revised to add Globalfiler and the 3500xL. Made manual CE Plate Prep a more clear option. Incorporated BAU for when Huntsville lab is authorized to perform procedure on casework.

Approval

Redacted - Signatures on File

Appendix A: Quality Control Procedures

1. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

The Amplification Kit Ladders are evaluated with the associated amplification kit. Refer to the nuclear DNA amplification procedure (i.e., DNA 213) for instructions.

2. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency and additional requirements.

A. Performance Verification (PV) of the Agilent Bravo Liquid Handler

1. An Artel MVS Multichannel Verification System and NIST traceable standards will be used to test the accuracy and precision of the liquid handling by the Agilent Bravo Liquid Handler. Refer to the *Artel MVS Multichannel Verification System User Guide* for operation of the Artel MVS.
2. The Agilent Bravo Liquid Handler workstations are configured with a ninety six (96) barrel pipette head and multiple volumes aliquoted during each procedure. A minimum of three repetitions (i.e., three plates) must be performed by the head for each volume to assess the accuracy and precision of the pipette head.
3. The results must be within the tolerance limits set by DCU for each volume. At times, it may be necessary to modify/optimize the Liquid Handler Liquid Class parameters (e.g., polynomial coefficient or pipette volume offset).
4. If the performance verification of the Agilent Bravo Liquid Handler does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

B. General Maintenance of the 3130xl Genetic Analyzer

The following recommended intervals and instructions provide guidance for the general maintenance of the 3130xl Genetic Analyzer and 3500xL Genetic Analyzer to include instruction for changing the capillary array and performing a spatial calibration or a spectral calibration. The Applied Biosystems 3130/3130xL Genetic Analyzers *Getting Started Guides* and *Maintenance, Troubleshooting, and Reference Guides* and 3500/3500xL Genetic Analyzer *User Guide* may be referenced for additional guidance.

General Maintenance	3130xl Recommended Interval	3500xL Recommended Interval
Replace Polymer	Weekly	14 days (or as required by usage)
Replace 1x Buffer/Water/ Waste	Weekly	
Replace Buffer (Anode and Cathode)		14 days (or as required by usage)
Install New Array	As needed	As needed
Water Wash	Weekly	
Flush Water Trap	Weekly	
Flush pump chamber and channels		Weekly
Flush pump trap		Monthly
Database Cleanup	Quarterly	Quarterly
Data Backup	Quarterly	Quarterly
Disk Defragmentation	Quarterly	Quarterly
Spatial Calibration	With array change or as needed	With array change, if detection cell window is opened, or as needed
Spectral Calibration	As needed or Quarterly	With array change or as needed
GS 600 LIZ v2 Sensitivity Evaluation		Semiannually or after optical adjustment

I. General Maintenance of the Applied Biosystems 3130xl

1. Prepare a new bottle of POP-4
 - a. Loosen the POP-4 bottle cap and allow it to sit on the bench top for approximately 15 minutes to degas.
2. Flush the polymer delivery pump (PDP)
 - a. Run the **water wash wizard** and use reagent grade water to flush the PDP.
Note: For a warm water wash, heat water to < 60°C.
 - b. Follow wizard prompts.
3. Flush the PDP water trap
 - a. Use a 20 mL Luer lock syringe filled with reagent grade water.
 - b. Attach the syringe to the forward facing Luer fitting at the top of the pump block, open the Luer approximately one-half turn counter clockwise.
 - c. Open the exit fitting at the top left side of the pump block approximately one-half turn counter clockwise.
 - d. Flush the water trap with approximately 5 mL of water
 - e. Close both fittings by turning them clock wise until finger-tight, do not over tighten.

II. General Maintenance of the Applied Biosystems 3500xL

1. Flush the pump chamber and channels
 - a. In the Maintenance Wizards screen, select **Wash Pump and Channels** and follow prompts to include the replacement of the polymer (POP-4) pouch.
2. Replace polymer (POP-4) pouch.
 - a. In Maintenance Wizards screen, select **Replenish Polymer** and follow prompts.
3. Replace Anode Buffer Container (ABC) and Cathode Buffer Container (CBC)
 - a. Allow refrigerated buffers to equilibrate to room temperature prior to first use. Do not remove seal.
 - b. Invert the ABC, then tilt slightly to make sure most of the buffer is in the larger side of the container. There should be less than 1mL of the buffer remaining in the smaller side of container.
 - c. Verify that the buffer is at the fill line, remove seal and place the ABC into the Anode end of the instrument.
 - d. Tilt the CBC back and forth gently to ensure that the buffer is distributed evenly across the container and that the buffer is at or above the fill line.
 - e. Remove the seal from the CBC, wipe off any excess buffer and place appropriate septa on both sides of the CBC.
 - f. Install the CBC on the autosampler. The CBC will click into the autosampler as the tabs are snapped into place.
 - g. Close the instrument door and click Refresh on the Dashboard to update status after changing the buffers.

III. Data Maintenance for the 3130xl and 3500xL

3130xl	3500xL
<ol style="list-style-type: none"> 1. Maintenance of the storage databases used by the Data Collection software. <ol style="list-style-type: none"> a. Open the appropriate results group folder and create a new backup folder using the naming convention, CE #XX_Backup_MMDDYY. b. Move all plate folders into the newly created backup folder and then copy it to the appropriate CE Backup folder on the network. 2. Delete records from the database <ol style="list-style-type: none"> a. From the navigation pane, select Database Manager and Cleanup Processed Plates. b. Allow the software the appropriate amount of time to delete the associated records and close the dialog box once 	<ol style="list-style-type: none"> 1. Maintenance of the storage databases used by the Data Collection software. <ol style="list-style-type: none"> a. Open the appropriate results group folder and create a new backup folder using the naming convention, CE #XX_Backup_MMDDYY. b. Move all plate folders into the newly created backup folder and then copy it to the appropriate CE Backup folder on the network. 2. Archive records from the database <ol style="list-style-type: none"> a. From the navigation pane, select Archive b. When prompted, enter the desired date range to be archived c. Specify the storage location to save the archive file d. Move the archive folder to the appropriate

complete. 3. Defragment the data storage hard drive using the disk defragmenter in Windows system tools to defragment the (E:) drive.	CE Backup folder on the network 3. Purge the previously archived records a. From the navigation pane, select Purge b. When prompted, enter the same date range as the Archive 4. Defragment the data storage hard drive using the disk defragmenter in Windows system tools to defragment the (E:) drive.
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IV. Array Change and Spatial Calibration

The capillary array will be changed as needed. The determination to change the array will be based upon a review of the quality of the data generated by the instrument. Generally, the array on the 3500xl should be changed after 160 injections. Be careful not to leave fingerprints on the array detection window.

3130xl	3500xL
1. From the toolbar select the Install Array Wizard . 2. Install the array as instructed by the wizard. a. Ensure the proper type (16-capillary) and length (36 cm) is entered in the array information fields. 3. In the final step of the wizard you can choose to fill the array with polymer or click finish if the array will be filled during the spatial calibration.	1. From the Maintenance Wizards screen, click Install Capillary Array 2. Install the array as instructed by the wizard. a. Ensure the proper type (24-capillary) and length (36 cm) is entered in the array information fields. 3. In the final step of the wizard you can choose to fill the array with polymer or click finish if the array will be filled during the spatial calibration.

A spatial calibration must be performed whenever a new array is installed. For a 3500xl, spatial calibration must be performed whenever the detection cell window is opened.

3130xl	3500xL
1. Select Spatial Run Scheduler in the navigation pane. 2. Select SpatialFill_1 . SpatialNoFill_1 can be selected if there is no need to fill the array with fresh polymer. 3. Click the Start button to initiate the spatial calibration. 4. Select Accept to accept the spatial calibration if the following criteria are met: a. Peaks of the spatial calibration are approximately the same height. b. An orange cross appears at the top (apex)	1. Select Maintenance in the navigation pane then select Spatial Calibration 2. Select Fill to fill the array with polymer before starting the calibration 3. Select Perform QC Checks 4. Click Start Calibration button to initiate the spatial calibration. 5. Select Accept Results to accept the spatial calibration if the following criteria are met: a. Peaks of the spatial calibration are approximately the same height.

of each peak in the profile. c. No irregular peaks are contained in the profile. d. RFU values for the peaks are greater than 2,000. e. The values for the Left Spacing and Right Spacing columns are 13-16 pixels. f. A spatial calibration can be accepted if one or more of the spacing values lie outside of this range but it is preferable to have all the values within this specification. The spatial calibration may be repeated as necessary.	b. One marker(a cross) appears at the top (apex) of each peak in the profile. c. No irregular peaks are contained in the profile d. RFU values for the peaks are greater than 3000 for a 24-capillary 3500xl array. e. Uniformity or peak height similarity values are 0.2 f. The values for the Capillary spacing are 2 pixels
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V. Spectral Calibration

A spectral plate may be reinjected or used for multiple instruments, of the same type, within a 24 hour period. A spectral calibration is generally run as needed for a 3500xL (e.g., decrease in spectral separation, new dye set, optical adjustment). A spectral calibration is required after changing the capillary array on a 3500xl.

3130xl	3500xL
1. Combine 195 µL of formamide with 5 µL of DS-33 Matrix Standard. 2. Dispense 10 µL of solution into the first two columns (wells A1-H1 and A2-H2). 3. Spin down and denature plate on thermal cycler then place on instrument. 4. Click Plate Manager in the navigation pane. Note: A previously created spectral plate can be duplicated by highlighting a plate in the Plate Manager window and clicking "Duplicate". This method will only require a new plate name be entered while retaining all the previously entered information. 5. Select New and the New Plate Dialog dialog box will open, fill out fields: a. Name: Use the naming convention CE#XX_Spectral_MMDDYY. b. Select Spectral Calibration from the Application drop down menu. c. Complete the remaining fields and select OK . This will open the "Spectral Calibration Plate Editor" window.	1. Combine 294 µL of formamide with 6 µL of DS-36 Matrix Standard (J6 Dye Set). 2. Dispense 10 µL of solution into the first three columns (wells A1-H1, A2-H2, & A3-H3) 3. Spin down and denature plate on thermal cycler then place on instrument 4. Access the Spectral Calibration screen, Select Maintenance , then select Spectral Calibration in the Navigation pane 5. Select number of wells on the plate (e.g., 96 well plate) and specify plate position on instrument 6. Select the chemistry standard and dye set for the calibration plate 7. Select Allow Borrowing . 8. Click Start Run Pass Criteria: The data collection software indicates the pass/fail status of each capillary. The spectral calibration is acceptable if the following criteria are met, and there is proper separation between the color channels.

<p>6. Create sample sheet.</p> <ol style="list-style-type: none"> Fill out the Sample Name fields to mirror the plate layout. Select Spectral_G5 from the drop down in the Instrument Protocol 1 field. Press OK to save plate sample sheet. <p>7. Select Run Scheduler from the navigation pane</p> <ol style="list-style-type: none"> Search the plate name or select find all and click on the plate to be run in order to highlight it within the list. Click Link to associate the sample sheet to the plates on the instrument. <p>8. Click the green arrow to start processing the spectral plate.</p> <p>9. The Data Collection software indicates the pass/fail status of each capillary. Review the spectral profile and raw data of each passing capillary. It is recommended that < 3 capillaries fail and no more than 2 in a row. The spectral plate may be reinjected if necessary. The Data Collection software automatically applies a saved spectral and no further action is required by the user.</p>	<ol style="list-style-type: none"> All capillaries have to meet the spectral Quality Value and Condition Number limits. The passing Quality Value for J6 Dye Set is a minimum of 0.95. The passing Condition Number value is a maximum of 8.0 for J6 Dye Set. ≤ 3 adjacent-capillary borrowing events allowed <p>The software gives a pass/fail status to each capillary. The user must evaluate the spectral profile traces and Accept Results or Reject Results.</p>
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C. Performance Verification of the 3130xl Genetic Analyzer and the 3500xL Genetic Analyzer

The performance verification of the 3130xl should be assessed with positive control DNA 9947A (0.10 ng/μL) amplified with Identifiler® Plus. The performance verification of the 3500xL should be assessed with positive control DNA 007 (0.10 ng/μL) amplified with Globalfiler.

3130xl	3500xl
<ol style="list-style-type: none"> Prepare a CE daughter plate for a single (16-capillary) injection typically including 14 replicates of amplified 9947A sample and 2 allelic ladders. A single plate may be used to evaluate multiple 3130xl instruments. Run the plate on the appropriate 3130xl instrument(s) and evaluate the data. A genetic analyzer is deemed suitable for casework analysis if: 	<ol style="list-style-type: none"> Prepare a CE daughter plate for a single (24-capillary) injection typically including 21 replicates of amplified 007 sample and 3 allelic ladders. A single plate may be used to evaluate multiple 3500xL instruments. Run the plate on the appropriate 3500xL instrument(s) and evaluate the data. A genetic analyzer is deemed suitable for casework analysis if:

<p>a. Correct and interpretable typing results are obtained for all successful injections of the positive amplification control DNA</p> <p>b. No allelic peaks, other than those attributable to the positive amplification control, are detected</p> <p>c. An appropriate sensitivity of detection is achieved.</p> <p>i. The sensitivity of detection is generally acceptable when the average peak height of each locus is between 850-1800 RFU. These values represent the lowest (Amelogenin) and highest (TH01) average values observed at a locus during the establishment of the 3130xl sensitivity of detection relative to the Match Interpretation Threshold as part of the Identifiler® Plus Amplification Kit at 27 cycles validation. Minor differences in average RFU values relative to the targeted sensitivity may be acceptable.</p> <p>ii. At the direction of the Technical Leader, the injection voltage of an individual 3130xl may be adjusted to maintain the instrument's sensitivity of detection. Generally, replicates of amplified control DNA 9947A are injected at the voltage that is currently in use, as well as higher and lower voltages, to determine the average sensitivity (per locus) under each injection condition. These averages are compared to the targeted RFU values.</p> <p>4. If the performance verification of the 3130xl does not meet the above criteria, the performance verification will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.</p>	<p>a. Correct and interpretable typing results are obtained for the positive amplification control DNA or ladder in at least one injection for each capillary.</p> <p>b. No allelic peaks, other than those attributable to the positive amplification control, are detected</p> <p>c. An appropriate sensitivity of detection is achieved.</p> <p>i. The sensitivity of detection is generally acceptable when the average peak height of each locus is between 1900-6800 RFU. These values represent the lowest (Th01) and highest (D8S1179) average values observed at a locus during the establishment of the positive control QC parameters during validation. Minor differences in average RFU values relative to the targeted sensitivity may be acceptable.</p> <p>ii. The sensitivity of detection is generally acceptable when the average peak height for the 11 GS600 LIZ peaks used for normalization is between 1700-5100 RFU for all the successful injections.</p> <p>4. If the performance verification of the 3500xL does not meet the above criteria, the performance verification will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.</p>
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The following are the 11 peaks used for normalization:

200	220	240	260	280	300	314	320	340	360	400
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D. Qualification of GS600 LIZ v2 Internal Size Standard

1. Prepare a CE daughter plate for the 3500xL using the instructions in section C and the new lot of GS600 LIZ.
2. Run the plate on an appropriate 3500xL instrument.
3. Analyze the data without normalization.
4. The sensitivity of the new lot will be accepted if the average allelic peak heights for the 11 GS600 LIZ peaks used for normalization (listed above) is between 1700-5100 RFU for all the successful injections (with a minimum of 16 of the 21 wells).
5. If the lot of GS600 does not meet sensitivity expectations, the assessment will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

E. GS600 LIZ v2 Sensitivity Evaluation

1. Prepare a CE daughter plate for the 3500xL using the instructions in section C. A single plate or multiple plates may be used to evaluate multiple 3500xL instruments. Run the plate(s) on the appropriate 3500xL instrument(s). A plate prepared for or data generated from section C may also be used for this evaluation.
2. Analyze the data without normalization.
3. The sensitivity of each instrument will be accepted if the average peak heights for the 11 GS600 LIZ peaks used for normalization (listed above) is between 1700-5100 RFU for all the successful injections.
4. If one or more instruments do not meet sensitivity expectations, the evaluation for that instrument will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

Procedures for the Interpretation of Nuclear DNA Typing Results from the Yfiler™ PCR Amplification Kit

1 Scope

These procedures apply to DNA personnel who verify and interpret nuclear DNA typing results obtained from the Yfiler™ PCR Amplification Kit using the GeneMapper™ ID-X (GMIDX) DNA typing software for forensic comparison purposes and the Y-Chromosome Haplotype Database for statistical analysis.

2 Background

Y-STR examinations are generally conducted for lineage comparisons, in support of Missing Persons and Intelligence cases, or on samples that have a high ratio of female to male DNA.

Y-STR profiles are considered haplotypes so all conclusions regarding comparisons apply to the person of interest (POI) and the paternal lineage of the POI. In addition, unrelated individuals may exhibit the same Y-STR typing results.

3 Equipment/Materials/Reagents

GeneMapper™ ID-X software (Applied Biosystems, version 1.5 or higher)

Y-Chromosome Haplotype Reference Database, current release, www.yhrd.org (Institute of Legal Medicine, Charite – University Medicine Berlin)

4 Standards and Controls

Raw data for the electrophoretic runs of samples or controls displaying no typing results must be reviewed for the presence of a primer peak. If no primer peak is observed, the sample must be reinjected or reprepared to verify that amplicon was added to the capillary electrophoresis (CE) plate.

4.1 Verification of GeneScan®-500 (GS-500) Internal Size Standard (ISS)

4.1.1 For Yfiler™ data, which is sized using the Local Southern Method, verify that the 75, 100, 139, 150, 160, 200, 300, 340, and 350 base pair (bp) fragments are captured and have been assigned the correct size values for each sample, control, and allelic ladder. In addition, the 400 bp fragment must be captured and properly assigned for any sample containing an allele ≥ 340 bp to be used for interpretation. Due to the temperature sensitivity of the 250 bp fragment's sequence-based conformation, this fragment is not used for sizing purposes.

4.1.2 If all of the GS-500 fragments for a given injection do not meet these specifications, a different injection of the sample that displays the correct size values for all of the GS-500 fragments must be used for interpretation of the entire DNA profile, which may require that the sample be reprocessed.

4.2 Verification of Allelic Ladders

4.2.1 Any allelic ladder used for genotyping must: 1) exhibit the correct allele designations (see Table 1) and 2) yield the correct typing results when used to genotype the positive amplification control.

4.2.2 If any sample(s) requires reinjection the appropriate ladder must be included in the reinjection set.

Locus ¹	Known Size Range (bp) ²	Alleles Present in Ladder ³	Color
DYS456	104-123	13-18	Blue
DYS389I	142-163	10-15	Blue
DYS390	191-227	18-27	Blue
DYS389II	252-293	24-34	Blue
DYS458	130-155	14-20	Green
DYS19	175-210	10-19	Green
DYS385a/b ⁴	242-317	7-25	Green
DYS393	100-131	8-16	Yellow
DYS391	150-175	7-13	Yellow
DYS439	197-225	8-15	Yellow
DYS635	246-270	20-26	Yellow
DYS392	290-325	7-18	Yellow
Y GATA H4	121-142	8-13	Red
DYS437	182-197	13-17	Red
DYS438	223-248	8-13	Red
DYS448	280-324	17-24	Red

Table 1 – Yfiler™ Allelic Ladder Specifications

¹ Yfiler™ haplotypes consist of tetranucleotide repeats with the exception of the trinucleotide repeat DYS392, pentanucleotide repeat DYS438, and hexanucleotide repeat DYS448.

² Sizes in base pairs are approximate due to electrophoretic variation and are based on plus-A addition. These sizes are published in the *AmpFISTR® Yfiler™ PCR Amplification Kit User's Manual* (Applied Biosystems, Inc.).

³ Ranges of alleles (i.e., 13-18) include only integers (i.e., 13, 14, 15, ..., 18).

⁴ DYS385 is a multi-copy (a/b) locus and may exhibit a single allelic peak or two different allelic peaks.

4.3 Positive Amplification Control (i.e., 007)

4.3.1 One positive control must be processed for amplification in parallel with each set or batch of evidentiary samples.

4.3.1.1 If any sample(s) requires reparation, the positive control and the appropriate ladder must also be reprepared.

4.3.2 If a set of samples has multiple injections of the positive control, at least one injection must exhibit all the expected allelic peaks ≥ 50 relative fluorescence units (RFUs) and must not exhibit any extraneous allelic peaks. A positive control with a non-allelic peak(s) (e.g., stutter, spike, pull-up) may be interpreted. See Table 2 for the expected positive control typing results using Yfiler™:

Locus	007 Control
DYS456	15
DYS389I	13
DYS390	24
DYS389II	29
DYS458	17
DYS19	15
DYS385a/b	11,14
DYS393	13
DYS391	11
DYS439	12
DYS635	24
DYS392	13
Y GATA H4	13
DYS437	15
DYS438	12
DYS448	19

Table 2 – Expected Yfiler™ STR Typing Results for the 007 Positive Control

4.3.2.1 Refer to the appropriate DNA procedure for the interpretation of nuclear DNA typing results (i.e., DNA 233) for guidance if the positive amplification control does not exhibit the expected results.

4.4 Negative Amplification Control

Refer to the appropriate DNA procedure for the interpretation of nuclear DNA typing results (i.e., DNA 233) for guidance on the evaluation of the negative amplification control.

4.5 Extraction Control (i.e., reagent blank)

Refer to the appropriate DNA procedure for the interpretation of nuclear DNA typing results (i.e., DNA 233) for guidance on the evaluation of the extraction control.

5 Procedures

5.1 DNA Profile Determination

5.1.1 Computer Assisted Allele Designations

The GMIDX software, using the analysis method settings represented in Appendix A, analyzes the data generated by the CE instruments and generates electropherogram data to be evaluated and interpreted. A pink box surrounding a data point label indicates that the software has identified a data point as an artifact. The GMIDX software uses the terms “spike” and “OMR” (Outside Marker Range) to represent a variety of DNA artifacts. Peak labels may be edited according to this procedure. Peaks interpreted as non-allelic may be deleted within GMIDX and will appear on the electropherogram with a single strikeout.

5.1.1.1 Identification of Peaks of Non-Genetic (Non-Allelic) Origin

Before the Y-STR typing results from a sample can be used for comparison purposes, it is necessary to identify any non-genetic peaks that do not represent allelic Y-STRs. These non-genetic peaks may be undesired PCR products (e.g., stutter, minus-A, and non-specific product), analytical artifacts (e.g., spikes and raised baseline), instrumental limitations (e.g., matrix failure), or be introduced into the process (e.g., dissociated primer dye and non-specific peaks).⁵

Reproducible non-genetic peaks (e.g., stutter, non-template dependent nucleotide addition, dissociated dye, matrix failure, non-specific product) may be interpreted.⁶ Non-reproducible non-genetic peaks (e.g., spikes and raised baseline) must be evaluated as specified.

Refer to the appropriate DNA procedure for the interpretation of nuclear DNA typing results (i.e., DNA 233) for information regarding excessive DNA template and off-scale samples, raised sample baseline, non-template-dependent nucleotide addition, matrix failure resulting in pull-up, spikes, and dissociated primer dyes.

5.1.1.1.1 Stutter

The kit-specific stutter percentage guidelines provided in Table 3 are estimates (Average + 3 SD)

⁵ The GMIDX software applies two levels of filtering to the sized Y-STR allelic data. The first filter is global and removes labels from peaks that are less than 2% of the peak height of the largest allele present at each Y-STR locus. The second filter removes labels from peaks at any locus that meet the FBI-defined sizing and relative peak height criteria for stutter and/or minus-A.

⁶ For purposes of interpreting DNA typing results, a peak need only be identified as being of non-genetic origin.

of the maximum expected stutter values at each locus in the Yfiler™ amplification kit. These values are expressed as a percentage relative to the source allelic peak height (i.e., % Stutter). Stutter peaks have been observed up to +/- 3 repeat units away from the source allelic peaks in both the absence and presence of intermediate stutter peaks. If such atypical stutter peaks are due to excessive amounts of template DNA, the sample may be reamplified with less template DNA or reinjected for less time.

Locus	-1 Repeat Unit	+1 Repeat Unit
DYS456	16	6
DYS389I	10	6
DYS390	13	6
DYS389II	18	6
DYS458	14	6
DYS19	13	6
DYS385a/b	17	6
DYS393	14	6
DYS391	10	6
DYS439	10	6
DYS635	13	6
DYS392	15	8
Y GATA H4	11	6
DYS437	8	6
DYS438	5	8
DYS448	6	5

Table 3 – Maximum Expected Yfiler™ STR Loci Stutter Percentages

5.1.1.1.2 Non-Specific Peaks

- An N-2 peak is frequently observed at the DYS19 locus as an off-ladder (OL) allele. This artifact is generally reproducible.
- A reproducible minus-A peak of the N-4 stutter peak may occur at DYS437.

5.1.1.1.3 Additional peaks of non-genetic origin are described in the appropriate DNA procedure for the interpretation of nuclear DNA typing results (i.e., DNA 233).

5.1.1.2 Off-Ladder (OL) Alleles

If an allele fails to size within a defined allele category (e.g., a bin or a virtual bin), it must be assigned a size using the following criteria.

5.1.1.2.1 Any sample containing an OL allele may be re-injected.

5.1.1.2.2 An OL allele may be a microvariant that sizes between two ladder alleles. For

example, if an OL allele occurs between the 12 and 13 ladder alleles and is approximately 1 bp larger than the 12 allele, it is designated as 12.1; 2 bp larger is designated 12.2; and so on.

5.1.1.2.3 If an OL allele does not fall within the size range of any locus-specific ladder, which includes the flanking virtual bins, it should be associated with one of the two loci between which it falls.

- a. For single-source samples, if the OL allele is flanked by a locus with one peak and a locus with no peaks (with the exception of DYS385 a/b), the OL allele is assigned to the latter locus.
- b. Generally, if both loci between which an OL allele falls each display a single allele, the OL allele may be assigned to the locus closest in size.⁷
- c. If determination of the locus assignment is not possible, both loci that flank the OL allele must be deemed inconclusive for matching/statistical purposes.
- d. If the OL allele is smaller in size than the smallest respective virtual bin, or larger in size than the largest respective virtual bin, the number of repeats in the allele should be estimated.
- e. When loci are closely spaced on the x-axis of an electropherogram, an above or below OL allele may be observed within the size range of a flanking locus.

5.2 Contamination Assessment

Refer to the appropriate DNA procedure for the interpretation of nuclear DNA typing results (i.e., DNA 233) for guidance on the evaluation of contamination in samples or controls.

5.3 Application of Peak Height Thresholds to Allelic Peaks

5.3.1 The Analytical Threshold (AT) is 50 RFU. At all loci other than DYS385a/b, any allelic peak detected at or above the AT may be used for matching/statistical purposes.

5.3.2 The Hemizygous Interpretation Threshold (HIT) is 125 RFU and is an empirically determined parameter established specifically for the DYS385a/b locus. The HIT is used to evaluate potential allelic drop-out in a profile.

5.3.2.1 The Y-STR typing results at DYS385a/b may be used for matching/statistical purposes if two alleles are detected or if one allele is detected at or above the HIT. If only one allelic peak is detected below the HIT, the locus may be used only for exclusionary purposes. However, if Y-STR or autosomal STR typing results indicate the presence of DNA from more than one male contributor, then two alleles detected below the HIT at DYS385a/b may not be used for matching/statistical and/or exclusionary purposes.

5.3.3 If no allele is detected at a locus, a null allele may be declared at that locus if all allelic peaks at all other loci are at or above the HIT.

⁷ To facilitate the interpretation of OL alleles, the Examiner may consult a listing of such alleles recorded at http://www.cstl.nist.gov/div831/strbase/var_tab.htm.

5.4 Interpretation of Y-STR Typing Results

To the extent possible, DNA typing results from evidentiary samples will be interpreted before the comparison to any known samples, other than those of assumed contributors.

When there are multiple amplifications and/or injections for a given sample extract, generally the one that provides the most information will be used for reporting. Data where saturation interferes with interpretation may require that an alternative amplification/injection is used.

5.4.1 Peak Height Ratio Assessment

For loci that contain more than one allelic peak, peak height ratios can be used to associate two alleles to a common source (i.e., duplication) or to establish the presence of a DNA mixture. Peak height ratio assessments are generally not used in the interpretation of knowns and items that are expected to have originated from a single source, such as bones and alternate knowns. The alternate known profile may be obtained as a single-source or as a major contributor typing result.

5.4.1.1 Peak height ratios (PHR) are calculated by dividing the peak height of the allele with the lower RFU value by the peak height of the allele with the higher RFU value, and then expressed as a percentage.

5.4.2 Determination of the Number of Contributors to Y-STR Typing Results

5.4.2.1 A profile is generally considered to have originated from a single male individual if one allele (other than DYS385a/b) is present at all loci for which typing results were obtained.

5.4.2.2 A profile is generally considered to have originated from more than one male individual if two or more alleles are present at two or more loci, other than DYS385a/b. The classification of any DNA profile as a mixture must be based on an evaluation of the DNA profile in its entirety.

5.4.2.3 Peaks that exceed the expected stutter percentages must be evaluated considering:

- A peak significantly above the stutter percentage is more likely to be allelic.
- A peak at a small (<200 bp) locus where possible minor contributor types are expected has more potential to be allelic.
- A peak in an additive stutter position, which exceeds the negative stutter percentage but not the combined positive and negative stutter percentages, may be considered stutter.
- Other apparent peaks below the AT suggest that the peak is potentially allelic.
- If the sample is a reference sample and expected to be single source, then these peaks can confidently be called stutter if there is no other evidence of contamination.

5.4.2.3.1 For apparent single source samples, a peak in a stutter position that exceeds the

expected stutter percentage may be interpreted as a stutter peak for purposes of determining the number of contributors to the sample. Generally, this interpretation is limited to a single instance unless the peaks are at a large (>200 bp) locus and there are no other indications of a mixture (e.g., peak height imbalance, apparent peaks <AT).

5.4.2.3.2 For mixed samples, peaks that exceed the expected stutter percentage are generally considered allelic for purposes of determining the number of contributors to the sample.

5.4.2.4 Duplications

- If two alleles are present at any locus other than DYS385a/b, a duplication event may have occurred. To be declared a duplication, the alleles should have a PHR $\geq 40\%$.
- Because a duplicated allele is typically one repeat unit larger or smaller than the other allele, the presence of two alleles at a given locus that differ in size by more than one repeat unit is generally indicative of a mixed profile.
- The proximity of certain loci along the Y-chromosome allows for the simultaneous duplication of alleles at multiple loci. Generally, loci that are less than 1 Mb apart could potentially be duplicated together.⁸ See Appendix B for the relative positioning and distance (i.e., Mb) of the Yfiler™ loci on the Y-chromosome.
- A profile in which three allelic peaks are observed at a single locus, but in which no other typing results indicate the presence of a mixture, may be concluded to be a single-source profile possessing a tri-allelic locus.
- The presence of more than one allele at DYS438 and/or Y GATA H4 is generally indicative of a mixed profile.

5.4.2.5 The number of contributors to a mixture should be based upon greatest number of alleles detected per locus, and, because of the potential for duplication, should generally be observed at two or more loci (other than DYS385a/b). This is the initial estimate of the number of contributors to the sample.

5.4.2.6 Using the loci with the largest number of alleles, assess the ratio of contributors. Evaluate peak height imbalance and account for allele sharing to determine if the number of contributors should be increased.

5.4.2.7 Apply the general pattern of number of contributors and mixture ratio across the profile to determine if other loci are consistent with this pattern or if the number of contributors should be increased or decreased by one. Loci with more alleles will be the most informative for this assessment. Additionally, apparent peaks <AT may also be considered, especially for low level samples.

⁸ A Y-STR haplotype exhibiting duplication at loci DYS437, DYS439 and DYS389I/II has been observed (Butler, JM, Decker, AE, Kline, MC, Vallone, PM. 'Chromosomal Duplications Along the Y-Chromosome and Their Potential Impact on Y-STR Interpretation'. J For Sci (2005), 50(4), 853-859).

5.4.3 Deduced Single-Source Profiles Determined by Separation of Expected DNA Typing Results

5.4.3.1 For mixed typing results, when the presence of an individual's DNA in the sample can be reasonably expected, the Y-STR typing results from the assumed contributor should be separated from the other mixture results to facilitate identification of the foreign alleles.

5.4.3.2 If sharing of alleles among the conditional male known profile and an additional male is suspected at a locus, no separation of each individual's alleles is possible at that locus.

5.4.3.3 This approach can also be used when another known male is expected to have contributed biological material to the mixed profile (e.g., consensual male). If more than one known male contributor is expected, each individual's alleles should be subtracted from the profile.

5.4.3.4 This approach can also be applied to evidentiary items from which DNA is isolated by means of a differential extraction. In such situations, the single-source or major contributor typing results from one fraction may be used as a conditional known profile(s) applied to the complementary fraction.

5.4.3.5 Mixtures comprised of three or more individuals generally may only be used for exclusionary purposes. If an apparent distinguishable mixture remains after subtraction of the assumed contributor, consult with the Technical Leader (TL) for additional guidance.

5.4.4 Deduced Single-Source Profiles Determined from Distinguishable Mixtures

5.4.4.1 A distinguishable mixture is a Y-STR typing result from a sample for which alleles can be attributed to individual major/minor male donors. In order to determine major/minor contributors to a mixture, every locus must be distinguishable except for DYS385a/b.

5.4.4.1.1 When a mixed profile contains at least one allele above the AT at every locus (i.e., full profile), the following should be applied:

- a. The PHR of alleles at all loci that exhibit two alleles (with the exception of DYS385a/b) must be $\leq 55\%$ in order to assign a major contributor. When all loci meet this requirement, the allele displaying the greater peak height at each locus may be attributed to the major contributor.
- b. If only one allele is detected at a given locus, that allele may be attributed to the major contributor.

5.4.4.1.2 When a mixed profile fails to produce alleles above the AT at one or more loci (i.e., partial profile), the following should be applied:

- a. The PHR at all loci that exhibit two alleles (with the exception of DYS385a/b) must be $\leq 40\%$ in order to assign a major contributor. When all loci meet this requirement, the allele displaying the greater peak height at each locus may be attributed to the major contributor.

- b. If only one allele is detected at a given locus, that allele may be attributed to the major contributor.

5.4.4.2 Mixtures comprised of three or more individuals generally may only be used for exclusionary purposes. If a major contributor can be discerned from a mixture comprised of three or more individuals, consult the Technical Leader for guidance.

5.4.4.3 Due to the possibility that the minor contributor's alleles may be either shared by the major contributor (and thus masked) or potentially not detectable (i.e., <50 RFU), determination of the minor contributor profile may be possible at only some loci.

5.4.4.4 Generally, a multi-locus, mixed sample that contains one or more true minor contributors can be expected to display at least one allelic peak in a non-stutter position.

5.4.5 Interpretation of Y-STR Typing Results for Indistinguishable Mixtures

An indistinguishable mixture is a Y-STR typing result from a sample for which alleles cannot be attributed to individual donors. Indistinguishable mixtures may be used for exclusionary purposes only.

6 Reporting Y-STR Results and Conclusions

The results and/or conclusions for specimens subjected to DNA analysis will generally be reported in narrative form with tables for statistical information, when applicable.

6.1 Only single-source or deduced single-source Y-STR profiles may be used for matching or statistical purposes.

6.2 Indistinguishable mixtures are not suitable for matching purposes and may be used for exclusionary purposes only.

6.3 The number of contributors must be determined for mixtures used for comparison purposes.

6.4 An exclusion is declared when two single source or deduced single source profiles are different at one or more loci for direct comparisons, or at two or more loci for paternal lineage comparisons.⁹ Differences that could be explained by allelic dropout cannot be the basis for an exclusion.

6.4.1 For a mixture comparison, an exclusion is declared when the types of a reference item cannot be included in the types present at the corresponding loci of the mixture profile, considering the number of contributors and the potential for dropout. An exclusion for a direct mixture comparison requires differences at one or more loci, while an exclusion for a lineage

⁹ One mismatch is not exclusionary in paternal lineage comparisons due to the potential for mutation.

mixture comparison requires differences at two or more loci.

6.5 A paternal lineage comparison is declared inconclusive when profiles from putative male relatives from the same paternal lineage are found to differ at only one¹⁰ locus, considering the potential for dropout.

6.6 Based on the structure of a Y-STR locus, a single mutation can cause differences at one or more loci when comparing results from individuals in the same paternal lineage. For example, at DYS389I & II, a single mutation may result in differences at both the DYS389I and the DYS389II loci. Similarly, a single mutation may result in an apparent duplication at DYS437 as well as an apparent null at DYS448. These differences alone cannot be the basis of a paternal lineage exclusion, and such profiles must be reported as inconclusive.

6.7 For both direct and lineage comparisons, an inclusion is declared when profiles are found to be the same at all loci for which interpretable DNA typing results were obtained.

6.7.1 Each DNA association or inclusion must be clearly and properly qualified with either a statistic or a qualitative statement. A qualitative statement not based on a statistical calculation should be limited to situations in which the presence of an individual's DNA on an item is reasonably expected. The provenance of the sample must be established in the case record when statistics are not calculated.

6.7.1.1 The Y- Chromosome Haplotype Reference Database (YHRD) calculates 95% Upper Confidence Interval (95% UCI) values using several general United States population groups: African American, Asian, Caucasian, Hispanic, and Native American. For all samples, the LR's for African American, Caucasian, and Hispanic are considered for reporting. For samples that potentially originate from Native American populations, the Native American LR is also considered.

6.7.1.2 The single lowest LR value across all populations considered is reported, and it is generally truncated to two significant digits for reporting. However, if this value is less than 10, it is truncated to one significant digit for reporting.

6.7.1.3 The magnitude of the LR relates to the degree of support provided by the evidence under the tested hypotheses and assumptions. A qualitative statement will be reported based on the following table:

LR	Qualitative Equivalent
1	Uninformative
2 to <100	Limited support for Inclusion
100 to <10,000	Moderate support for Inclusion
10,000 to <1,000,000	Strong support for Inclusion

Table 4 – Qualitative Equivalent Scale for Y-STR Likelihood Ratios

¹⁰ For lineage comparisons, a single difference between two items may occur due to (a) mutation among relatives in the same paternal lineage or (b) different paternal lineages.

7 Calculations

7.1 Calculation of Statistics Using the Y- Chromosome Haplotype Reference Database (YHRD)

The primary database used for estimating the haplotype frequency is the Y-Chromosome Haplotype Reference Database (YHRD; <https://yhrd.org>), which is used to provide Likelihood Ratios from frequency estimates for African American, Caucasian, Hispanic, and Native American population groups, when applicable.

7.1.1 Go to the YHRD website (<http://yhrd.org>). Select the “Search the Database” tab. Whenever possible, for profiles where electropherograms reflect the searched profile, choose the “search using your ... GeneMapper® ID/ID-X...” option to import Y-STR profiles directly from an exported GMIDX file. Alternately, select “Manually enter the haplotypes/haplotypes...” (Figure 1).

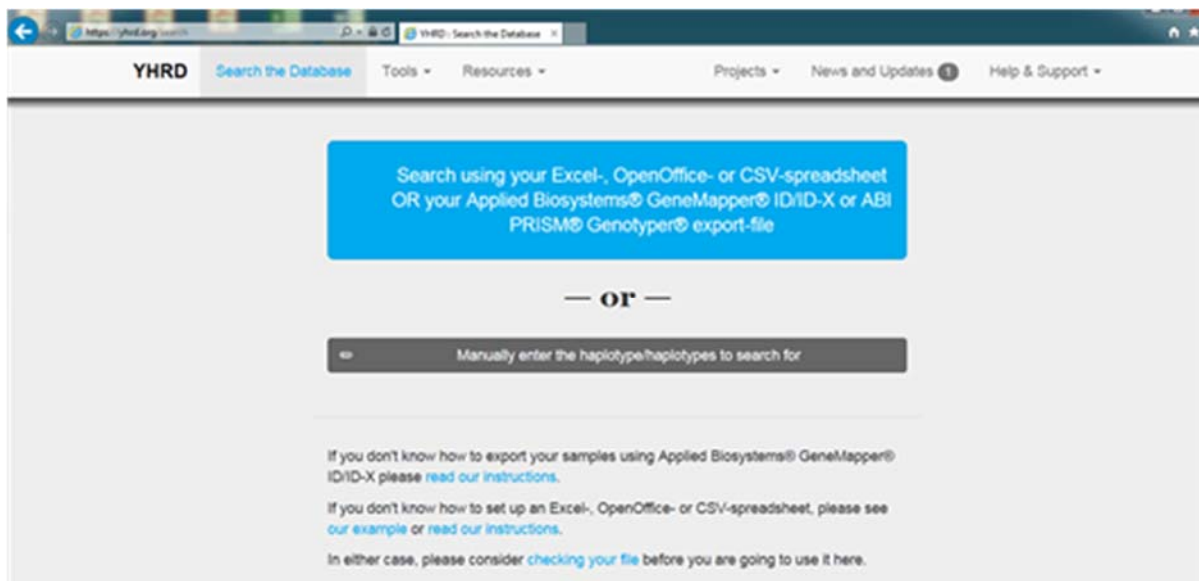


Figure 1 – YHRD Search the Database Page

7.1.2 Exported GMIDX files for STaCS entry can be used for import into YHRD. For electronically imported profiles, designate each profile to be searched, choose “Yfiler”, then select “Search”. (Figure 2)

YHRD Search the Database Tools Resources Projects News and Updates Help & Support

Heads up! We came across some issues while parsing your file. Carefully compare the data stated here with those of your original file. If you would like to know more about the issues we had with your file, consider [checking your file using our "Data File Validator"](#).

Minimal PowerPlex Y **Yfiler** PowerPlex Y23 Yfiler Plus Maximal

☒ 1

DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385	DYS393	DYS391	DYS439	DYS635	DYS392	YGATAH4	DYS437	DYS438	DYS448
16	13	26	31	16	14	12, 14	13	10	13	23	13	12	14	13	19

☒ 2

DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385	DYS393	DYS391	DYS439	DYS635	DYS392	YGATAH4	DYS437	DYS438	DYS448
16	13	26	31	16	14	12, 14	13	10	13	23	13	12	14	13	19

☒ 3

DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385	DYS393	DYS391	DYS439	DYS635	DYS392	YGATAH4	DYS437	DYS438	DYS448
16	13	26	31	16	14	12, 14	13	10	13	23	13	12	14	13	19

Search

Figure 2 – YHRD Electronic Data Import

7.1.3 For manually entered profiles, choose “Yfiler” and enter the alleles for the appropriate markers, then select “Search” (Figure 3).

YHRD Search the Database Tools Resources Projects News and Updates Help & Support

Minimal PowerPlex Y **Yfiler** PowerPlex Y23 Yfiler Plus Maximal

☒ Manual input

DYS456	DYS389I	DYS390	DYS389II	DYS458	DYS19	DYS385	DYS393	DYS391	DYS439	DYS635	DYS392	YGATAH4	DYS437	DYS438	DYS448

Search

Figure 3 – YHRD Manual Data Input

7.1.4 For each sample, select “Add feature to this report”, and choose “National Database (with Subpopulations, 2014 SWGDAM-compliant)”. Note that YHRD will only search against the database haplotypes that contain the same or more loci than were entered.

7.1.5 The number of observations of the searched haplotype is listed for each subpopulation.

7.1.6 For instances in which the haplotype has been observed in the database ($x > 0$), YHRD calculates a one-sided 95% confidence interval using the Clopper and Pearson formula:¹¹

¹¹ The Clopper and Pearson formula calculates the exact confidence interval. The exact confidence interval is a cumulative binomial distribution for all values from 0 to x matches given a sample of size n and frequency p . The listed formula is for the upper limit of a one-tailed confidence interval.

$$\sum_{k=0}^x \binom{n}{k} p_0^k (1 - p_0)^{n-k} = \alpha$$

where $k = 0, 1, 2, 3, \dots$ x observations; n = database size; and x = number of observations of the haplotype in the database. When $\alpha = 0.05$, solving for p_0 yields the population frequency of the upper bound of the 95% upper confidence limit. This value is the reported “95% UCI” in YHRD and is the profile probability: the probability of observing the haplotype after adjusting for sampling uncertainty.

7.1.6.1 For instances in which the haplotype has not been observed in the database, the following formula (derived with $x = 0$ in the Clopper and Pearson formula) is used to estimate the upper bound of the 95% confidence limit, p_0 :

$$p_0 = 1 - \alpha^{1/n}$$

where $\alpha = 0.05$ or 5% and n = database size. For example, if a particular haplotype is not observed in a database of 2000 haplotypes, then the 95% upper confidence limit is estimated to be $1 - (0.05)^{1/2000} = 0.0014977$ or 1 in every 778 haplotypes. Note that when $\alpha = 0.05$, p_0 is very close to $3/n$ (e.g., $3/2000 = 0.0015$).

7.1.7 The likelihood ratio (LR) of the upper bound frequency estimate describes how much more likely are the results if the matching profiles are from the same source or the same paternal lineage rather than if one profile is from an unknown, unrelated individual or lineage. The LR should be calculated:

$$LR = \frac{1}{95\% \text{ UCI}}$$

7.1.7.1 YHRD provides the 95% UCI expressed as 1 in X . The LR is the reciprocal of the 95% UCI, which simplifies to $LR = X$.

8 Suggested Reporting Language

The results and/or conclusions for specimens subjected to DNA analysis will generally be reported in narrative form. The formatting and administrative information required in a report are described in the appropriate *FBI Laboratory Operations Manual* practices and the *DNA Procedures Manual*. For guidance on reporting language for Introductory Statements, Amplification Kit Used, Comparisons to Previously Reported Results, Alternate Reference Samples, Elimination Samples, and Differentially Extracted Samples refer to the appropriate interpretation protocols of the *DNA Procedures Manual* (i.e., DNA 233).

8.1 Report Wording Examples

See section 8.3 for endnote language, denoted A-F in these examples.

Direct Comparisons

[Match]

The Y-STR typing results from item 1 were interpreted as originating from two individuals. The major contributor profile from item 1 is 1,100 times more likely if JAMES is the major contributor than if an unknown, unrelated male is the major contributor.^A

Person of Interest (POI)	Likelihood Ratio (LR) ^B	Level of Support ^C
JAMES	1,100	Moderate support for Inclusion

The following individuals are excluded as potential contributors to the Y-STR typing results obtained from item 1:^A

- JONES
- WHITE

[Uninformative]

The Y-STR typing results from item 1 were interpreted as originating from one individual. The Y-STR typing results from item 1 are equally likely^D if JAMES is the contributor than if an unknown, unrelated male is the contributor.^A

Person of Interest (POI)	Likelihood Ratio (LR) ^B	Level of Support ^C
JAMES	1	Uninformative

Lineage Comparisons

[Paternal Relative as Alternate Reference]

Information provided by [person, agency] identifies BROWN as the biological father of JAMES GARCIA. The Y-STR typing results obtained from Item 2 and BROWN are the same; therefore, item 2 could have originated from GARCIA.^A These results are 310 times more likely if item 2 is from GARCIA than if item 2 is from an unknown, unrelated male.

Likelihood Ratio (LR) ^B	Level of Support ^C
310	Moderate support for Inclusion

[Paternal Relatedness – Inclusion]

Information provided by [person, agency] identifies JOHNSON as the potential biological brother of MILLER. The Y-STR typing results from JOHNSON and MILLER are the

same; therefore, they could be biological brothers.^A These results are 960 times more likely if JOHNSON and MILLER are paternal relatives than if JOHNSON is from an unknown unrelated paternal lineage.

Likelihood Ratio (LR) ^B	Level of Support ^C
960	Moderate support for Inclusion

[Paternal Relatedness – Exclusion]

Information provided by [person, agency] identifies JOHNSON as the potential biological brother of MILLER. Based on the Y-STR typing results, JOHNSON is excluded as a biological brother of MILLER.^A

[Paternal Relatedness – Inconclusive]

Information provided by [person, agency] identifies SPARKS as the potential biological brother of MILLER. Based on the Y-STR typing results, no conclusion as to the possible biological relationship between SPARKS and MILLER can be made.^E

Assumed Contributors (e.g., intimate sample or consensual partner)

[No DNA Unlike]

The Y-STR typing results for item 3 indicate the presence of a single male individual. No Y-STR typing results unlike JONES were obtained from item 3; therefore, no comparisons were made to WHITE.

[Match to Deduced Profile]

The Y-STR typing results from item 3 were interpreted as originating from two individuals, one of whom is JONES. The Y-STR typing results unlike JONES are 230 times more likely if WHITE is the contributor than if an unknown, unrelated male is the contributor.^A

Person of Interest (POI)	Likelihood Ratio (LR) ^B	Level of Support ^C
WHITE	230	Moderate support for Inclusion

BROWN is excluded as a potential contributor to item 3.^A

Indistinguishable Mixture Comparisons

[Unsub]

The Y-STR typing results for item 1 indicate a mixture of male individuals and are suitable for comparison purposes. Because these mixture results cannot be attributed to individual contributors, they are not suitable for matching purposes; however, they may be used

for exclusionary purposes.

[Indistinguishable comparison]

The Y-STR typing results for item 1 indicate the presence of DNA from three male individuals. Because these mixture results cannot be attributed to individual contributors, they are not suitable for matching purposes; however, they may be used for exclusionary purposes. SMITH is excluded as a potential contributor of the DNA obtained from item 1.^A No conclusion can be provided for JONES.

8.2 Other Reportable DNA Typing Results

8.2.1 When no reference sample is provided for comparison, the results should be reported as follows:

“The Y-STR typing results for item 1 indicate a single male individual and are suitable for comparison purposes.”

“The Y-STR typing results for item 1 indicate a mixture of male individuals and are suitable for comparison purposes.”

8.2.2 For samples for which insufficient DNA is recovered for DNA typing, this information should be reported as follows:

“No Y-STR typing results^F were obtained from item 1; therefore, no comparisons could be made to SMITH.”

8.2.3 Y-STR typing results may be obtained that are suitable for entry into the Combined DNA Index System (CODIS) or other appropriate database. Refer to the appropriate DNA procedure (i.e., DNA 233) for reporting this information.

8.3 Associated Endnotes for Reporting Language

^A Barring mutation, any male relative within the same paternal lineage has the same Y-STR profile and would also be expected to be included/excluded as a potential contributor.

^B The likelihood ratio is a statistical approach that compares the probabilities of observing the DNA results under two alternative propositions. Calculations were performed using the African American, Caucasian, and Hispanic populations in the Y Chromosome Haplotype Reference Database (release xxx). The lowest calculated likelihood ratio is reported.

^C These likelihood ratio ranges provide the following support for Y-STR conclusions:

<u>Likelihood Ratios:</u>	<u>Qualitative Equivalent:</u>
1	Uninformative
2 to <100	Limited support for Inclusion
100 to <10,000	Moderate support for Inclusion
10,000 to <1,000,000	Strong support for Inclusion

^D This conclusion is drawn when the likelihood ratio is equal to 1; this comparison is uninformative.

^E A paternal lineage comparison is declared inconclusive when profiles from putative male relatives from the same paternal lineage are found to differ at only one locus, considering the potential that allele dropout occurred.

^F Insufficient DNA quality and/or quantity can affect the ability to generate a DNA typing result.

9 Sampling

Not applicable.

10 Measurement Uncertainty

Not applicable.

11 Limitations

11.1 These procedures do not exhaust the possible list of the results that may be encountered by the Examiner. For those results not specifically described, conclusions should be drawn using the procedures given for the results above that are similar in concept and/or origin.

11.2 It is sometimes necessary to consume a sample in its entirety to ensure that the best attempt possible is made to obtain DNA typing results for comparison purposes. Should the total consumption of a sample be required, an Examiner should obtain and record permission from the contributing agency or other responsible office prior to testing.

11.3 A paternal lineage consists of those male relatives to whom the same Y-chromosome has been transmitted from a common ancestor. Barring mutation, all male relatives within the same paternal lineage have the same Y-STR profile. Attribution of the Y-STR typing results to a single individual, to the exclusion of relatives in the paternal lineage, is not possible based on Y-chromosome loci. Additionally, unrelated individuals may exhibit the same Y-STR profile.

12 Safety

Not applicable.

13 References

DNA Procedures Manual

AmpF/STR Yfiler PCR Amplification Kit, User's Manual, Applied Biosystems, 2004 Part number 4358101 Rev. A.

Mulero, J.J., Chang, C.W., Calandro, L.M., Green, R.L., Li, Y, Johnson, C.L., and Hennessy, L.K. Development and validation of the AmpF/STR YFiler PCR amplification kit: a male specific, single amplification 17 Y-STR multiplex system, *J. Forensic Sci.* (2006) 51:64-75.

Clopper, C., Pearson, E. The use of confidence or fiducial limits illustrated in the case of binomial. *Biometrika* (1934) 26:404-413.

Ge, J., Budowle, B., Planz, J.V., Eisenberg, A.J., Ballantyne, J., and Chakraborty R. US forensic Y-chromosome short tandem repeats database. *Legal Med* (2010) 12:289-295.

Kayser, M., Brauer, S., Willuweit, S., Schadlich, H., Batzer, M.A., Zawacki, J., Prinz, M., Roewer, L., and Stoneking, M. Online Y-chromosomal short tandem repeat haplotype reference database (YHRD) for U.S. populations. *J. Forensic Sci.* (2002) 47:513-519.

Butler, J.M., Decker, A.E., Kline, M.C., and Vallone, P.M. Chromosomal duplications along the Y-Chromosome and their potential impact on Y-STR interpretation. *J. Forensic Sci.* (2005) 50:853-859.

Butler, J.M., Schoske, R. Duplication of DYS19 flanking regions in other parts of the Y chromosome. *Int. J. Legal Med* (2004) 118:178-183.

SWGDAM Interpretation Guidelines for Y-Chromosome STR Typing by Forensic DNA Testing Laboratories. Most current version. Available at SWGDAM.org/docs.html.

Rev. #	Issue Date	History
5	02/28/18	<p>Updated to DNA Procedures Manual throughout.</p> <p>1: Simplified Background information to remove specifics about data collection and interpretation. Specified inconclusive language for consistency with match and exclusion.</p> <p>3: Updated GeneMapper version.</p> <p>Relocated NA sections to end and renumbered remaining sections.</p> <p>Moved procedures for verifying standards and evaluating of control to Standards and Controls section.</p> <p>Updated reference to appropriate interpretation procedure throughout.</p> <p>5.1.1: Removed “off-ladder alleles” from list of items referenced in appropriate interpretation SOP.</p> <p>5.1.1.2: Added section on Off Ladder (OL) alleles.</p> <p>6: Changed header to “Suggested Reporting Language”. Updated report wording for known samples throughout section.</p> <p>6.1: Updated introductory statement and endnote for Y-STRs.</p> <p>6.5.3: Removed section for reporting also match results. Renumbered subsequent section.</p> <p>Removed section on reporting most discriminating statistic for concordant types, and no statistic reported for samples with autosomal source attribution, to reflect current practices.</p> <p>10.2: Clarified examiner role in consumption of evidence request.</p>
6	10/02/19	<p>Replaced Appendix A tables with GMIDX settings</p> <p>Entire document revised and reorganized for clarity.</p> <p>Statistical tool transitioned from US Y-STR database to YHRD.</p> <p>Report language updated to reflect YHRD statistics and to mirror autosomal DNA typing.</p> <p>Moved Y-chromosome figure to Appendix B.</p>

Approval

Redacted - Signatures on File

DNA Technical Leade

Date: 10/01/2019

DCU Chief

Date: 10/01/2019

SBAU Chief

Date: 10/01/2019

QA Approval

Quality Manager

Date: 10/01/2019

Appendix A: GMIDX Analysis Settings

Analysis Method Editor

General **Allele** Peak Detector Peak Quality SQ & GQ Settings

Bin Set: AmpFLSTR_Yfiler_Binset_v2

☒ Use marker-specific stutter ratio and distance if available

Marker Repeat Type:		Tri	Tetra	Penta	Hexa
Global Cut-off Value		0.02	0.02	0.02	0.02
MinusA Ratio		0.0	0.12	0.0	0.0
MinusA Distance	From	0.0	1.85	0.0	0.0
	To	0.0	2.04	0.0	0.0
Global Minus Stutter Ratio		0.0	0.0	0.0	0.0
Global Minus Stutter Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0
Global Plus Stutter Ratio		0.0	0.0	0.0	0.0
Global Plus Stutter Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0

Amelogenin Cutoff 0.0

Range Filter... Factory Defaults

Save As Save Cancel Help

Figure 1 - Allele Tab for YFiler™

Appendix A: GMIDX Analysis Settings (cont.)

Analysis Method Editor

General Allele **Peak Detector** Peak Quality SQ & GQ Settings

Peak Detection Algorithm: Advanced

Ranges

Analysis: Partial Range Sizing: Partial Sizes

Start Pt: 2200 Start Size: 75

Stop Pt: 9000 Stop Size: 400

Smoothing and Baseline

Smoothing: ☐ None ☒ Light ☐ Heavy

Baseline Window: 51 pts

Size Calling Method

☐ 2nd Order Least Squares
☐ 3rd Order Least Squares
☐ Cubic Spline Interpolation
☒ Local Southern Method
☐ Global Southern Method

Peak Detection

Peak Amplitude Thresholds:

B: 50 R: 50

G: 50 P: 50

Y: 50 O: 150

Min. Peak Half Width: 2 pts

Polynomial Degree: 3

Peak Window Size: 15 pts

Slope Threshold

Peak Start: 0.0

Peak End: 0.0

Normalization

☐ Use Normalization, if applicable

Factory Defaults

Save As Save Cancel Help

Figure 2 - Peak Detector Tab for YFiler™

Appendix B: Map of the Y-Chromosome

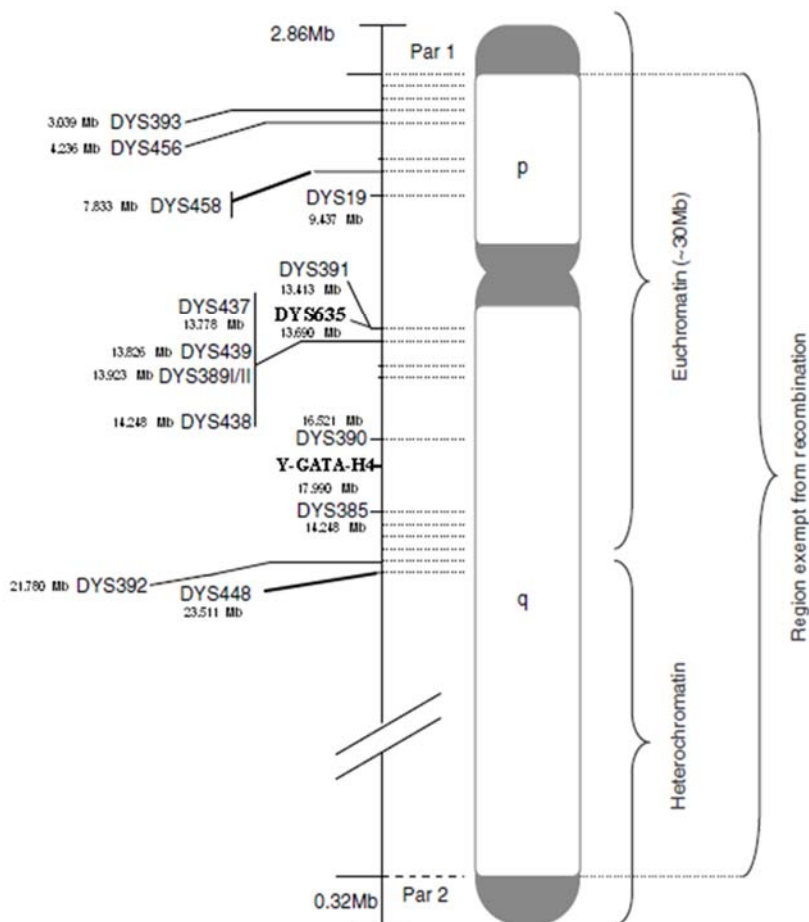


Figure 3 – Y-Chromosome with Yfiler™ loci and their relative locations and distances¹²

¹² Buckleton, J., C. Triggs and S. Walsh. Forensic DNA Evidence Interpretation. CRC Press; Boca Raton, FL, 2005.

Procedures for the Quantification of Human and Male Nuclear DNA

1 Scope

These procedures apply to DNA personnel who perform quantification to determine the quantity of amplifiable human and male nuclear DNA (nDNA) detected in a sample and DNA personnel that perform the associated quality control procedures. The DNA Casework Unit (DCU) and Scientific and Biometrics Analysis Unit (SBAU) use Sample Tracking and Control Software (STACS) and robotic workstations to automate the set-up of the quantification (aka quant) plates.

2 Equipment/Materials/Reagents

Equipment/Materials

- Tecan robot model 'Freedom EVO'
 - Tecan EvoWare software, version 2.3 or higher
- 7500 Real-Time PCR System, Applied Biosystems
 - HID Real-Time PCR Analysis Software vs 1.2 or higher
- STACS, version 3.2.920 or higher
- General laboratory supplies (e.g., pipettes, tubes)
- Microcentrifuge tubes (robot compatible)
- Speed-Vac, Vacufuge Concentrators, or equivalent
- 96-well Plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear plate seals
- Thermal Microplate Sealer

Reagents

- Quantifiler® TRIO DNA Quantification Kit
 - Quantifiler® TRIO DNA standard OR previously prepared dilution series
- Quantifiler® Automation Enhancer
- 007 sample, quantified and diluted as necessary
- TE Buffer (TE)
- 3% bleach (reagent grade or equivalent)
- 10% bleach (reagent grade or equivalent)
- Isopropyl alcohol, 70%
- Purified water or equivalent, available at laboratory sinks
- Water (reagent grade or equivalent)
- Roboscrub solution (Liquinox™ or equivalent)

3 Standards and Controls

The Quantifiler® TRIO DNA standard dilution series will be run in duplicate on each plate to generate the standard curve that is used to extrapolate the quantity of DNA in each sample.

A Master Mix control and TE control will be run on each plate. Evaluation of these standards and controls can be found in the Data Evaluation section of this procedure.

The 007 control is used as a positive control for troubleshooting purposes but there are no evaluation criteria for this sample.

The reagent blank(s) (RB) associated with each extraction batch are quanted to determine the RB with the greatest (if any) signal.

4 Procedures

Refer to the DNA Procedures Introduction (i.e., DNA QA 600) and follow applicable general precautions and cleaning instruction.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water will be used. The purified water, available via faucets (typically labeled DE) at the laboratory sinks, is used for Tecan operation and is also called Tecan system liquid.

4.1 Concentrating Extracted Samples Using the Speed-Vac

4.1.1	<p>Samples may be concentrated using a Speed-Vac.</p> <ul style="list-style-type: none"> • Samples from questioned items and corresponding RBs are generally reconstituted with reagent grade water, vortexed and quick spun prior to quantitation. Female fractions from vaginal swabs (and similar sample types) are generally not concentrated. • Known samples are generally not concentrated. <p>The volume of water used to reconstitute, typically 15 µL or 25uL, will be recorded in the case notes. This volume is determined by the type of sample or as requested by the examiner.</p> <p>The volume used for the RB must be the same or less than the volume used for the associated samples.</p>
-------	---

The Speed-Vac flask should be empty and dry, and the flask seal should be tight.

The Speed-Vac should be turned on ~45 minutes prior to use.

Ensure the gasket on the centrifuge is in its proper position and that the rotor is properly tightened prior to sample processing.

With the heat set to “High”, a 100 μ L extract may take ~1 hour dry. Samples should not be dried on “High” for more than four hours.

On the Speed-Vac with the heat set to “High”, a 50 μ L extract may take ~30-40 minutes to dry and a 100 μ L extract may take ~60 minutes to dry. Samples should not be dried on “High” for more than four hours (maximum starting volume of ~400 μ L).

On the Vacufuge with a setting of 60°C, a 50 μ L extract takes about 45 minutes.

4.2 Preparing the Tecan Robotic Workstation

If necessary, turn on the Tecan, which will undergo an initialization routine. Log on to the Tecan computer, launch and log on to the current Tecan software.

4.2.1	<p>Ensure the Tecan is prepared to run:</p> <p>Prior to daily use:</p> <ul style="list-style-type: none"> • Make ~100mL of 3% bleach to replace in front trough. • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script <p>Prior to each run:</p> <ul style="list-style-type: none"> • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. <i>When a carboy is refilled, it should be allowed to de-gas overnight before use.</i> • Check volume of waste container and empty if needed <p>As needed:</p> <ul style="list-style-type: none"> • Clean barcode scanners with a lint-free cloth 	
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The daily start up script prompt “Check syringes and tips” refers to checking that the tubing and syringes (plunger lock screws) are tight and not introducing air bubbles, and that the tips are tight, free of clogs, and not leaking.

4.3 Preparing the Sample Rack and Creating a Scan File Import

Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

Ensure all DNA extracts and reagent blanks (aka DNA sample tubes) are in Tecan compatible tubes and appropriately barcoded. Ensure all tubes are uncapped prior to run.

4.3.1	<p>Place DNA sample tubes in positions 1 through 16 in the sample racks. Use additional sample racks as needed (up to 6 racks or 83 sample tubes). Any rack position(s) unfilled by a DNA sample tube must contain an empty tube with a unique “BL” barcode.</p>	
--------------	--	--

“BL” barcode tubes may be reused; however, each “BL” barcode on the Tecan must be unique.

4.3.2	Use the current appropriate script to scan the sample racks and generate a .csv scan file. Import the file into STACS.	
--------------	--	--

4.4 Master Mix Preparation

This step may be performed any time prior to loading the master mix on the Tecan robot.

4.4.1	Enter appropriate barcodes into STACS. Create the master mix based on the volumes below. Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin.	
--------------	---	--

Quantifiler TRIO Master Mix Components

	μL per well*
PCR Reaction Mix	10.0
Primer Mix	8.0
Automation Enhancer	0.018**

*Number of wells = number of samples + 10 standards, 3 controls, and appropriate overage (~6)

NOTE: Master mix must be created for a minimum of 56 wells to prevent pipetting less than 1 ul of automation enhancer.

**Round the total volume of automation enhancer to 2 decimals as appropriate for the pipette capability.

4.5 Preparing the Tecan Deck

The below steps may be performed in any order prior to running the Tecan robot.

Positions of materials may vary between instruments. The robotic script will direct the placement.

4.5.1	Bleach/TE Rack: <ul style="list-style-type: none"> Ensure the 3% bleach solution in the front trough was replaced prior to first daily use. If making new standards, replace the ~100 mL TE in the center trough. Ensure that the trough has a “TE” barcode and is replenished as needed. 	
--------------	---	--

4.5.2	<p>If using the Tecan to reconstitute samples: Water Rack:</p> <ul style="list-style-type: none"> • Ensure center trough has ~200 mL reagent grade water, replaced prior to each run. 	
4.5.3	<p>Place tubes in the Standards Rack (see Figure 1):</p> <ul style="list-style-type: none"> • Positions 1 through 6: the Quantifiler® TRIO stock DNA standard solution* (with a unique “BL” barcode) and the standard dilution series tubes (with barcodes “S1” through “S5”). • Positions 7 through 14: empty tubes (with unique “BL” barcodes). • Position 15: a new uncapped tube (with a “TE” barcode). • Position 16: an uncapped 007 tube (with an “MC” barcode) 	

Before loading, vortex, quick spin, and uncapped the Quantifiler® TRIO stock DNA standard and/or the prepared standard dilution series, as appropriate.

Ensure the date the standard dilution series is prepared and biologist’s initials are recorded on the standards (or standard rack). Prepared Quantifiler® TRIO standard dilution series may be used a maximum of five times or up to 5 days. The standards (or standard rack) will be labeled to indicate the number of times they have been used and refrigerated between uses.

*If using a previously prepared standard dilution series, any tube with a unique “BL” barcode (to include the stock standard tube) may be placed in position 1.

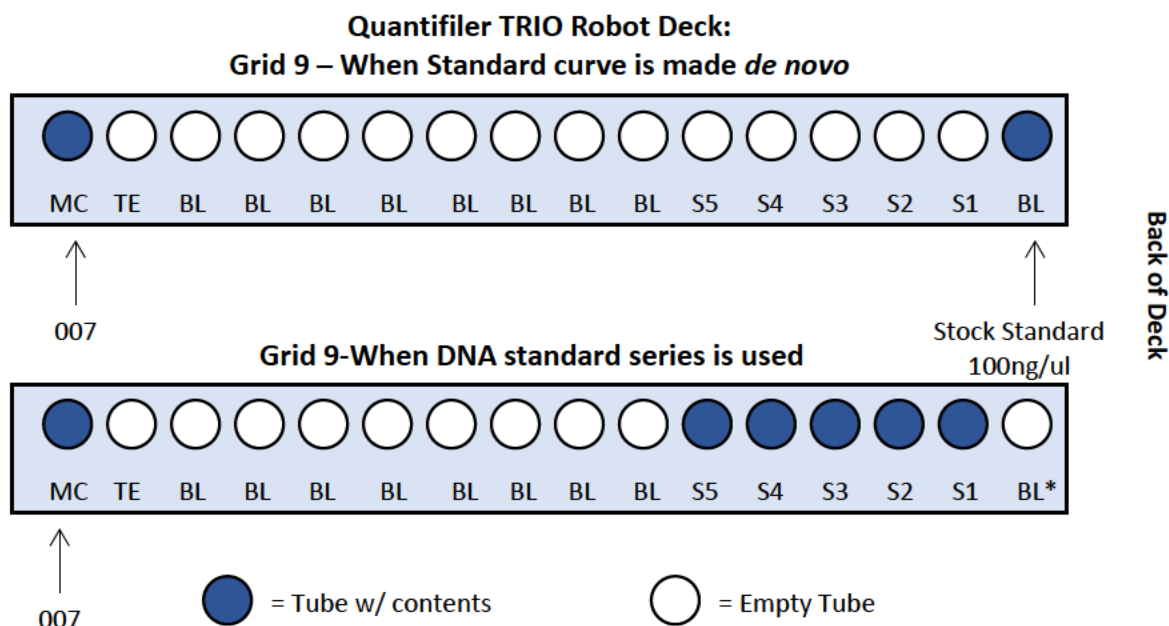


Figure 1 – Example Sample Positioning for Standards Rack

4.5.4	Plate Rack: <ul style="list-style-type: none"> Place a 96-well plate into a base. Place into the front position of the plate rack with the A12 notch at back right. Ensure a quant batch barcode label is on the right side of the base or the plate, as appropriate. 	
4.5.5	Place tubes in the Master Mix Rack (see Figure 2): <ul style="list-style-type: none"> Positions 3 and 4: the two tubes containing equal volumes of master mix (with “C1” barcodes). Ensure tubes are uncapped. Positions 1, 2, and 5 through 16: empty tubes (with unique “BL” barcodes). 	

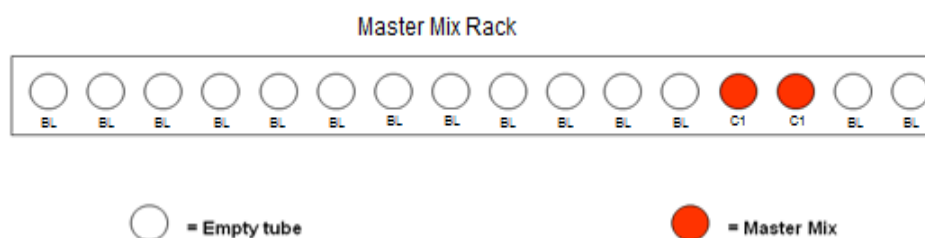


Figure 2 –Positioning for Master Mix Rack

4.6 Tecan Plate Preparation

4.6.1	Run the current version of the FBI Quantifiler TRIO script, and answer the prompts. The Tecan will add 18 μ L of Quantifiler® TRIO master mix and 2 μ L of each sample extract or control to the 96 well plate. The run takes ~20 minutes.	
4.6.2	Seal the plate with a clear seal. Quick spin (generally ~2,000 rpm for 5 seconds). Ensure the quant plate barcode is on a side of the plate.	

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are well sealed.

The DNA sample tubes and standard dilution series tubes should be removed from the Tecan deck and capped prior to taking the sealed quant plate to the Amp room.

4.7 Real-Time PCR

4.7.1	Ensure the 7500 and the supporting computer are powered on. Place the sealed plate into the 7500 so that well A1 is in the back-left and the notched corner of the plate is in the back-right.	
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4.7.2	In the 7500 software, open a new run file. Import the sample setup (.txt file) generated by STACS for the plate ID.	
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The .txt file can be found in the appropriate folder on the network.

4.7.3	Save the run file with the plate ID in the file name, ensure the 7500 door is closed, and start the run.	
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Start the run from the Instrument tab or the Instrument menu.

The 7500 run generally takes ~1 hour.

4.8 Data Evaluation

4.8.1	Review the results in the 7500 software.	
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The data may be analyzed from Amplification Plot of the Analysis Menu.

4.8.2	Review the Standard Curve plots of C_T (cycle threshold) versus Quantity (DNA concentration). [See Figures 3-5.] Use the Target dropdown menu to view the standard curve results for each target.	
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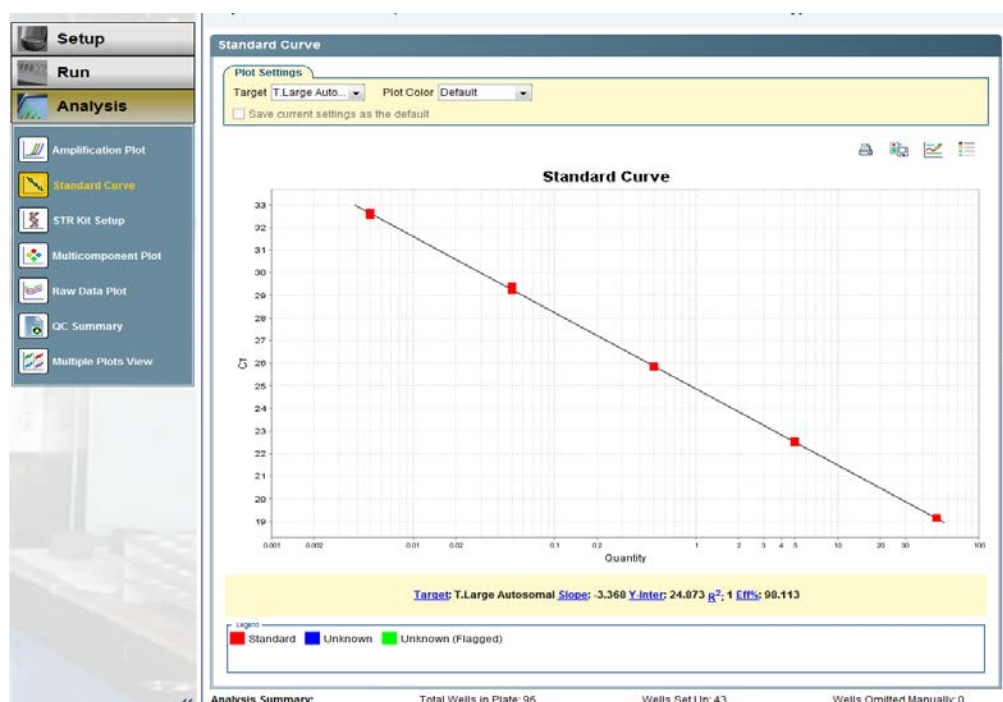


Figure 3 – Typical Standard Curve Demonstrating T. Large Autosomal TRIO Results

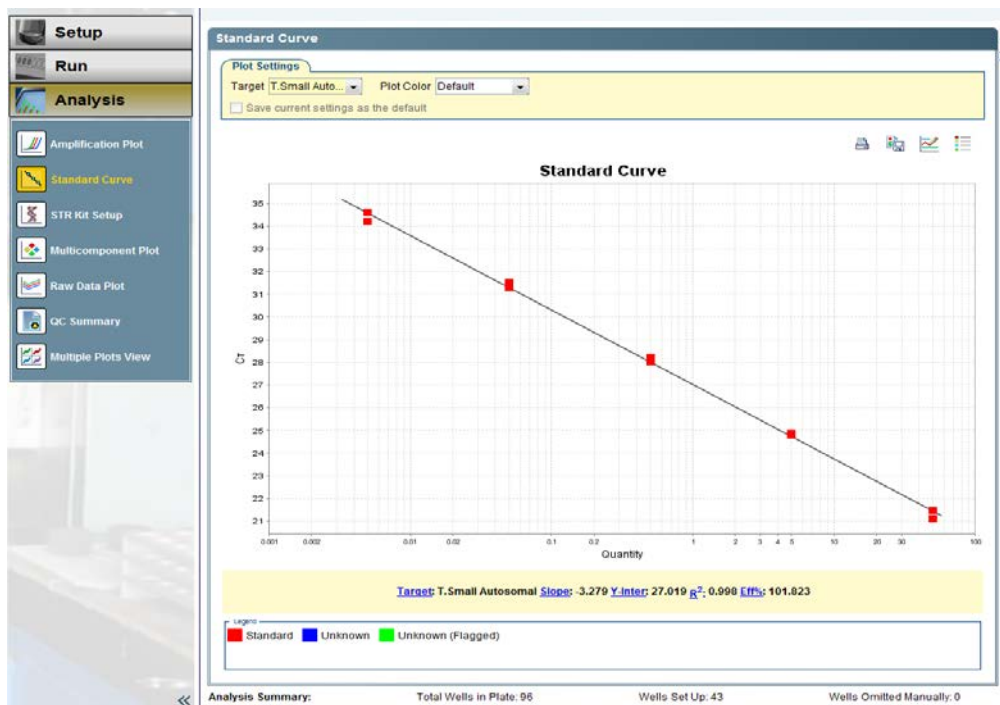


Figure 4 – Typical Standard Curve Demonstrating T. Small Autosomal TRIO Results

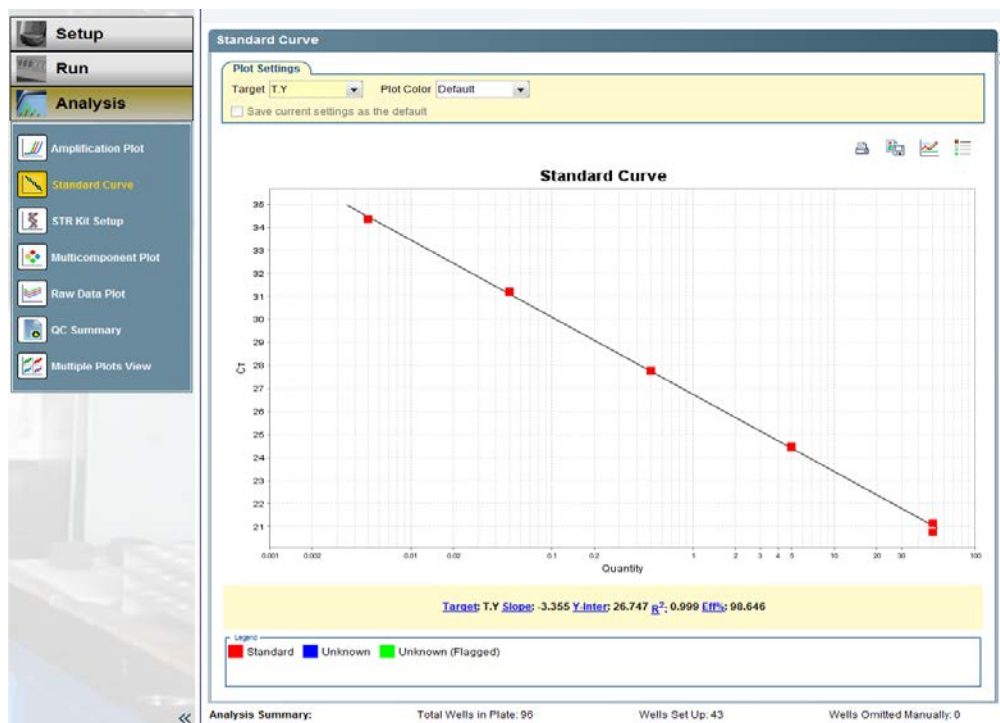


Figure 5 – Typical Standard Curve Demonstrating T.Y TRIO Results

4.8.2.1	A passing run will meet the standard curve parameters below:	
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	Slope Range		Y-intercept Range		R² Minimum
T. Large autosomal	-3.664	-3.185	23.496	25.892	0.997
T. Small autosomal	-3.435	-3.115	25.678	27.716	0.996
T. Y	-3.574	-3.116	24.697	27.296	0.995

4.8.2.2	If the Y-intercept, R ² , or slope are out of range, or if there is a visible outlier, omitting a poor replicate of a standard and reanalyzing may improve the standard curve. (For each standard pair in the dilution series, only one of the replicates may be omitted if necessary).	
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4.8.2.3	If the Y-intercept, R ² , and/or slope do not meet the required values after replicate omission, the plate fails, the data is not suitable for evaluation, and the samples must be re-quantified.	
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If one or more replicates are deleted from the standard curve, or the Y-intercept, R², or slope fall outside the expected range, the standard dilution series used should be discarded.

4.8.3	Export the results to the appropriate file on the network. (<i>Select Export from File menu, then choose Results.</i>) Import the results file (.txt) into STACS. Enter the plate ID and the requested values for the T. Large Autosomal, T. Small Autosomal and T.Y assays (i.e., Y-intercepts, slopes, and R ² s) into STACS. This data transfer should be checked to ensure accurate transcription and who performed the check recorded in the notes. Import the .eds file into STACS.	
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The tabulated data can now be reviewed in STACS.

4.8.4	Check the T. Large Autosomal, T. Small Autosomal and T.Y quantification results and IPC C _T of the Master Mix control. The Master Mix control should display no quantifiable DNA. If a DNA concentration value appears in the master mix control, the concentration values obtained for the RB(s) run on the plate should be examined. <ul style="list-style-type: none"> If one or more of the RBs display no quantifiable DNA, the master mix value can be concluded to be spurious (i.e., not indicative of the presence of adventitious DNA) and the sample data should be used. The T. Large Autosomal target is not used for quantification. Values appearing in this target alone should not be considered. 	
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4.8.5	<p>For plates using a new DNA Standard dilution series, check the T. Small Autosomal and T.Y quant result and IPC C_T of the TE Control. The TE control should display no quantifiable DNA.</p> <ul style="list-style-type: none"> • If a DNA concentration of ~0.010 ng/μL or less is detected, the sample data should be used. • If a DNA concentration between ~0.010 and 1 ng/μL is detected, the samples may be re-quanted. • If a DNA concentration of ~1 ng/μL or greater is detected, the samples must be re-quanted. 	
4.8.6	<p>The sample data can be evaluated to determine if any sample should be diluted and/or re-quanted. IPC C_T values are typically between 27 and 30. Undetermined IPC C_T values or values greater than 31 may indicate inhibition.</p> <ul style="list-style-type: none"> • Samples that have an indication of possible inhibition may be diluted and re-quanted. • Samples with excessive DNA (generally >300 ng/μL) should be diluted and re-quanted. 	

Reagent grade water is used to dilute samples as appropriate. Any dilution(s) made will be recorded in the case notes.

An examiner will review the quant results for each sample. STACS uses the quant results and the default amplification settings to determine the volume of sample to queue for amplification. An examiner should make adjustments to the amplification setup as necessary. Additional guidance is located in the nDNA amplification procedure in the *DNA Procedures Manual*.

5 Calculations

Not applicable.

6 Sampling

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

Based on internal validation studies, samples that fail to yield signal at quant will not yield amplification results. In rare instances, Trio may be affected by inhibition when amp kits are not. In such cases, it is possible that samples yielding no result at quant may yield DNA typing results.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

9.2 Avoid reaching into the Tecan robot while it is running as personal injury could result from moving robot accessories.

10 References

FBI Laboratory Quality Assurance Manual (QAM)

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. *Quantifiler™ HP and Trio DNA Quantification Kits User Guide*, 2017.

Applied Biosystems. *Applied Biosystems 7500/7500 Fast Real-Time PCR System Getting Started Guide for Relative Standard Curve and Comparative CT Experiments*. 2010.

Applied Biosystems. *Installation and Maintenance Guide for the Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System*. 2006.

ARTEL. *MVS Multichannel Verification System User Guide*. 2006

Alfonina I, Zivarts M, Kutuyavin I, et al. 1997. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. *Nucleic Acids Res.* 25:2657-2660.

Green RL, Ines CR, Boland C, and Hennessy LK. 2005. Developmental validation of the Quantifiler real-time PCR kits for the quantification of human nuclear DNA samples. *J. Forens. Sci.* 50:809-825.

Higuchi R, Dollinger G, Walsh PS, and Griffith R. 1992. Simultaneous amplification and detection of specific DNA sequences. *BioTechnology* 10:413-417.

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Livak KJ, Flood SJ, Marmaro J, Giusti W, and Deetz K. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. *PCR Methods Appl.* 4:357-362.

Rev. #	Issue Date	History
4	05/25/16	Complete revision for simplification of procedure. Changed from nDNAU to DCU throughout. Changed from nDNAU LIMS to STACS throughout and made necessary adjustments for STACS. Added 4.1 from extraction SOP since performed during quant. Moved QC procedures to Appendix and simplified.
5	02/28/18	1 Adjusted scope 2 Updated software and made necessary adjustments throughout 4.1.1 and 4.1.2 Allowed for both volumes typically used. Added clarification that volume is based on sample or as requested by FE. 4.5.4 Corrected numbering and added allowance for different placement of the barcode based on the Tecan plate holder. 4.8.2.1 Applies to human and male standard curves Appendix A: Edits so that standards in performance verification can be run in duplicate.
6	03/16/20	Complete revision to incorporate changes from Quantifiler DUO to Quantifiler TRIO. Moved RoboScrub to appendix A

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 03/13/2020

DCU Chief

Date: 03/13/2020

SBAU Chief

Date: 03/13/2020

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency and additional requirements.

A. General Maintenance of the AB 7500 Real-Time PCR System

Once a year, general maintenance is performed as part of the annual PM. For semi-annual general maintenance, refer to the instructions in the Applied Biosystems *7500/7500 Fast Real-Time PCR System Maintenance Guide* to perform the following:

1. Regions of Interest (ROI) Calibration (Chapter 2)
2. Background Calibration and Optical Calibration (Chapter 3)
3. Dye Calibrations (Chapter 4) for standard dyes VIC and FAM and custom dyes ABY and JUN and, when applicable, for standard dye NED used for the mtDNA qPCR Degradation Assay.

B. Performance Verification of the AB 7500 Real-Time PCR System

The performance verification of the AB 7500 Real-Time PCR System will be accomplished by running both the Quantifiler® TRIO DNA Quantification Kit and, when applicable, the mtDNA qPCR Degradation Assay, as each assay uses different dyes.

1. Refer to the above Quantifiler® TRIO procedures and the procedures for the mtDNA qPCR Degradation Assay (i.e., DNA 404):
 - a. Using an in-use lot of Quantifiler® TRIO kit, run a plate containing the standard dilution series and appropriate controls, all in duplicate.
 - b. Using in-use lots of reagents for the mtDNA qPCR Degradation Assay, run a plate containing the mtDNA Quantitative PCR Standard Dilution Series, the HL60 calibrator, and appropriate controls, all in duplicate.
2. The 7500 will be deemed suitable for casework analysis if
 - a. The slope, Y-intercept, and R^2 values for the Quantifiler® TRIO meet the criteria of a passing run:

	Slope Range		Y-intercept Range		R^2 Minimum
T. Large autosomal	-3.664	-3.185	23.496	25.892	0.997
T. Small autosomal	-3.435	-3.115	25.678	27.716	0.996
T. Y	-3.574	-3.116	24.697	27.296	0.995

- b. And the slope, Y-intercept, and R^2 values for the mtDNA qPCR Degradation Assay meet the criteria of a passing run:
 - i. $R^2 \geq 0.985$
 - ii. Slope in the range of -3.200 and -3.600
 - iii. Y-intercept in the range of 36.100 and 39.600

3. If the performance verification of the 7500 does not meet the passing criteria for either assay, the unsuccessful plate(s) will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

C. General Maintenance of the Tecan Robotic Workstation

RoboScrub cleaning should be performed weekly, generally at the end of a workday:

1. Make ~3.5 L of diluted Liquinox (see instructions on the label of the bottle for preparation)
2. ~3.5 L purified water in a separate container is needed
3. Run the RoboScrub Clean script, and follow the prompts

D. Performance Verification of the Tecan Robotic Workstation

1. An Artel MVS Multichannel Verification System and NIST traceable standards will be used to test the accuracy and precision of the liquid handling by the Tecan. Refer to the *Artel MVS Multichannel Verification System User Guide* for operation of the Artel MVS.
2. The Tecan Robotic workstations are typically configured with eight (8) fixed tips and there are multiple volumes aliquoted during each procedure. A minimum of 6 repetitions will be performed with each tip for each volume.
3. The results must be within the tolerance limits set by DCU for each volume. At times, it may be necessary to modify/optimize the Tecan liquid class parameters (e.g., offset and factor).
4. If the performance verification of the Tecan does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

A. Qualification of Applied Biosystems Quantifiler® TRIO DNA Quantification Kit

Each new lot of Quantifiler® TRIO kits will be evaluated by running a standard dilution series from the new lot in duplicate with appropriate controls. The new Quantifiler® TRIO kit lot will be deemed suitable for casework analysis if the slope, Y-intercept, and R^2 values meet the passing criteria in section 4.8.2.1.

Executive Summary

Title: Validation of the Quantifiler™ Trio DNA quantification kit

Purpose: To evaluate the use of Quantifiler Trio for quantification of male and human DNA, and to characterize the relationship between Trio values and Globalfiler amplification results.

Redacted

Initiation date: August 29, 2017

End date: November 8, 2019

Background:

Currently, the FBI Laboratory's DNA Casework Unit (DCU) and Scientific & Biometrics Analysis Unit (SBAU) use the Life Technologies (ThermoFisher) Quantifiler Duo realtime PCR kit to quantify the concentration of human genomic DNA and the male component in extracts before nuclear DNA analysis is performed. The Quantifiler Trio kit, also by Life Technologies, introduces new reporter dyes designed to increase spectral resolution and sensitivity. It also targets two genomic amplification fragments of differing lengths, allowing an assessment of DNA degradation, which will not be used by the FBI due to its previously established unreliability when analyzing low quantity samples. This validation assessed the Quantifiler Trio kit in accordance with the SWGDAM guidelines for internal validation. In addition to identifying the sensitivity and limitations of the kit, this study served to determine the acceptable operating parameters of the standard curve, to characterize the relationship between Trio quantification and Globalfiler amplification, and to ensure that the use of automated systems for reaction setup is reliable and repeatable. Finally, the data collected here were used to evaluate the feasibility of a 'zero is zero' processing policy in which no amplification is attempted if a zero quant is obtained.

Supporting Documentation: Associated documentation and data files are located on the DSU\Projects\Trio folder

Validation summary:

Accuracy and precision were first tested by pipetting standards across a plate and analyzing them as samples. As standards are designed to be of a known quantity, they should yield an expected measurement when analyzed as samples. A high **accuracy** of the kit was confirmed with a percent error (actual value compared to expected value) of only 1.53 when standards were measured as samples. The **precision** of the kit was tested by quantifying a collection of 24 known samples in triplicate, yielding a coefficient of variation (variation about the mean) of only 0.04, meaning that on average, the standard deviation was only 4% of the mean.

Contamination was evaluated by three experiments in which plates were prepared on DCU Tecans. The first plate, which was designed to detect environmental contamination, contained 83 blanks and yielded quantifiable signal ≤ 0.0004 ng/ul in 11 wells. However, when amplified using Globalfiler and analyzed with a PAT of 50 RFU, no alleles were detected. The second and third tests consisted of zebra and checkboard plates, respectively, and were designed to test for sample carryover on the tips of the Tecan. Nine blanks yielded signal on the zebra plate and 3 yielded signal on the checkerboard, none of which were greater than 0.0009 ng/ul. Again, when amplified using Globalfiler, no alleles were detected. The extreme sensitivity of Trio appears to result in sporadic signal, but in practice such signal does not result in data when amplified.

Sensitivity of the Trio kit was evaluated through the quantification of two male samples and a female sample across a dilution range from 10 ng/ul to 0.0001 ng/ul. The results of the experiment showed that Trio was capable of consistently detecting DNA at the lowest levels tested. Quantifiler Duo, in comparison, appeared to have a lower limit of detection of 0.001 ng/ul. When amplified, it became clear that Trio is far more sensitive than Globalfiler. When amplifying samples below 0.001 ng/ul, no alleles were detected when analyzed with a PAT of 150 RFU. When samples above 0.01 ng/ul were amplified, full profiles were obtained. The results of this study support the implementation of a 'zero is zero' policy and indicate that an even higher quantification limit (such as 0.0005) may be appropriate with further analysis of casework-derived data. It is important to note that this determination is based on an amplification input volume of 10 ul. If future protocols incorporate an option to amplify 15 ul of input volume, a new sensitivity study will be required.

Testing of **case-type samples** yielded data in agreement with the sensitivity study, showing that samples that quantify below 0.001 ng/ul did not yield data when amplified with Globalfiler. Unlike the sensitivity study however, a measurement of 0.02 ng/ul was required before a full profile was obtained with Globalfiler. Low level samples were collected from personal items and showed that Trio was capable of detecting a male minor contributor when Duo was not.

Mixtures were generated using two males and a female, and covered a range of ratios from 1:1 to 1:200, with each male being represented as either the major or minor contributor, for a total of four sets. The results showed that in samples less than 1 ng/ul, Trio is capable of detecting a male minor contributor as low as 1:200 (1:183 program-calculated), while Duo failed to detect one male below 1:20 and the other below 1:50. When amplified, Globalfiler was unable to detect the male minor at ratios greater than 1:100, again demonstrating that Trio is more sensitive than Globalfiler.

Analysis of 30 standard curves generated over the course of the validation allowed for the establishment of acceptable operating parameters. For the human small target, the slope should be between -3.435 and -3.115, the Y-intercept should be between 25.678 and 27.716 and R^2 should be ≥ 0.996 . For the human large target, the slope should be between -3.664 and -3.185, the Y-intercept should be between 23.496 and 25.892 and R^2 should be ≥ 0.997 . For the male target, the slope should be between -3.574 and -3.116, the Y-intercept should be between 24.697 and 27.296, and R^2 should be ≥ 0.995 . A month-long **stability** study was conducted to determine how long a Tecan-prepared standard curve would meet these operating parameters and showed that prepared standards should be discarded after 5 days.

In order to evaluate the sensitivity of the kit to **inhibition**, and to compare its response to that of Globalfiler, five inhibitors commonly encountered in case-type samples were introduced at varying

concentrations into the quant/amp workflow. With the possible exception of the blue dye indigo, the results showed that Trio was equally or less susceptible to inhibition than Globalfiler.

Site specific studies including assessments of contamination, sensitivity, and precision were performed in Huntsville and all results fell within the expected ranges established during validation at Quantico.

Conclusion:

This validation study has shown the Quantifiler Trio kit to be extremely sensitive and highly reproducible. In fact, this kit is at least 10 times more sensitive than the currently used Quantifiler Duo kit and can detect a male minor contributor at a level four times lower. Because the kit is significantly more sensitive than the Globalfiler amplification kit, a casework processing policy of 'zero is zero' is considered valid based on the data generated here.

Procedures for Familial Comparison and Interpretation Using the KIn CALc Kinship Software (v. 5.0.10_FBI)

1 Scope

These procedures describe the methods by which DNA typing results are interpreted for familial comparison purposes using kinship analysis software, KIn CALc.

2 Background

Upon completion of the technical aspects of DNA analysis, the DNA typing results must be first verified and interpreted by an Examiner using the methods established in the appropriate interpretation standard operating procedure (SOP) of the *DNA Procedures Manual*.

Based on the transmission of genetic material within a family, DNA typing results can potentially be used to establish the likelihood of biological relatedness. DNA typing results obtained from potential relatives (e.g., Unidentified Human Remains [UHRs] and relatives of a missing person, an alleged parent and a child) may be compared and used in statistical kinship assessments using the kinship analysis software KIn CALc. Based on manual comparisons of the DNA typing results (e.g., autosomal STR, Y-STR, mitochondrial DNA testing), an Examiner may be able to determine whether an individual can be excluded as a potential biological relative, and a kinship calculation is not necessary.

KIn CALc is an Excel-based program that allows the user to evaluate a putative familial relationship, given the DNA typing results of a “Test” sample and other “Reference” sample(s). The Test sample may be a sample from evidence (e.g., a UHR sample) or a known reference sample for which the relationship to the other known reference samples is in question (e.g., paternity analysis). The software is used to calculate a likelihood ratio (LR) or combined kinship index (KI) from multiple population databases. The KI conveys the ratio of the probabilities of observing the DNA profiles under two mutually exclusive hypotheses: (1) that the Test and Reference(s) are biologically related in the manner assessed and (2) generally that the Test and Reference(s) are unrelated. Generally a KI greater than one supports the hypothesis of relatedness and a KI less than one supports the alternate hypothesis, generally, of unrelatedness. The pedigree assessment is based on the information provided by the contributor. Requests for additional permutations of the pedigree must be approved by the Technical Leader (TL). These conclusions are compiled by the Examiner into a written report and are the official FBI Laboratory findings as to the nuclear DNA typing results.

3 Equipment/Materials/Reagents

KIn CALc Software Version 5.0.10_FBI

Redacted

4 Analytical Procedures

When a manual comparison of the results excludes the potential relationship (see section 5.1.4), use of the KIn CALc software is not necessary.

4.1 Software-assisted Kinship Calculations

4.1.1 Determination of Hypotheses

The primary hypothesis is the probability of observing the DNA results if the Reference(s) is (are) biologically related to the Test. The alternate hypothesis is generally the probability of observing the DNA results if the Reference sample(s) is (are) unrelated to the Test. The Examiner will determine the type of relationship to be assessed based on the case information (e.g., the Reference samples are from the parents of a missing person, and the Test is from a UHR which is potentially that missing person).

4.1.2 Kinship Calculation using KIn CALc

4.1.2.1 Theta

The theta value is by default set to 0.01 for kinship analysis using KIn CALc. This value should be used when assessing the relationship likelihoods in African American, Caucasian, Southwestern Hispanic, Southeastern Hispanic, Filipino, Trinidad or Chamorro populations. Theta should be manually changed to 0.03 when determining the likelihood of a relationship in Native American populations (Apache, Navajo or Minnesota).

4.1.2.2 Linkage

4.1.2.2.1 Two pairs of loci are linked closely enough to affect the KI in some situations:¹

- CSF1P0 and D5S818
- D12S391 and vWA

4.1.2.2.2 For simple paternity cases (i.e., a paternity duo or trio where the TEST is the alleged parent) and for simple reverse paternity cases (i.e., a reverse paternity duo or trio where the TEST is the alleged child), all loci may be used in the KI calculation if results are available.

4.1.2.2.3 Both loci of a linked pair must not be used in the KI calculation for pedigrees that are not simple paternity or simple reverse paternity.² The locus used in the calculations should be the more discriminating of the pair. If results are only available for the less discriminating locus,

¹ Despite their location, there is no evidence of linkage disequilibrium for the aforementioned markers at the population level due to, presumably, the re-assortment of alleles throughout sufficient generations. Therefore, these markers can be considered 'independent' for calculations involving unrelated individuals.

² There are additional specific situations in which it would be appropriate to use both loci of a linked pair. To avoid these complexities, a more conservative approach is used for the purposes of this SOP: only one locus of a linked pair will be used in KI calculations unless the assessment is for paternity or reverse paternity.

it may be used in the KI calculation.

- CSF1P0 is more discriminating than D5S818
- D12S391 is more discriminating than vWA

4.1.2.3 Input of DNA Profile Information

GlobalFiler™ (GF) profiles must be analyzed with AT150 to be assessed using the KIn CALc software, i.e., Globalfiler AT50 profiles cannot be evaluated with the KIn CALc software. Identifiler® Plus (ID+) profiles must be analyzed at AT50 to be assessed with the KIn CALc software. Any locus with only one allele with a peak height less than the respective stochastic threshold (ST) (i.e., 725 RFU for GF and 200 RFU for ID+) must not be entered into KIn CALc. These loci are inconclusive for KI calculations. For single source profiles, if a locus has two alleles, both alleles may be entered into KIn CALc regardless of peak height.

4.1.2.3.1 Option 1: Manual Entry

4.1.2.3.1.1 Open the KIn CALc software and navigate to the “Kit Conversion” tab.

4.1.2.3.1.2 Enter the sample identifier for the “Test” sample (e.g., item 5) under the cell labeled “Item #” in the “Commercial Multiplex Format” column (yellow column). For paternity cases the “Test” will always be the alleged parent. Enter the DNA profile results obtained for the Test.³ **In the KinCalc software, the alleles must always be entered in increasing numerical order for each locus (e.g., 11, 12 not 12, 11). Additionally, if a locus is homozygous, the allele must be entered into both the “Allele 1” and “Allele 2” rows.** Once all genetic data is entered, select the “Insert Test Profile” button. See Figure 1.

4.1.2.3.1.3 Enter the sample identifier for the first Reference under the cell labeled “Item #” and enter the DNA profile results obtained for the Reference in the cells corresponding to the loci for which data is available.⁴ Once complete, click the “Insert AR1 Profile” button.

4.1.2.3.1.4 If multiple reference samples are available, repeat step 4.1.2.3.1.3, selecting the appropriate “Insert AR# Profile” button, until the information for all samples has been entered.

4.1.2.3.1.5 Once all references are entered, click the “To Pedigree” or “To Custom Pedigree” button as appropriate or manually navigate to the appropriate tab.

³Only alleles that comply with the procedures set for use in statistical analysis will be entered. Refer to the appropriate interpretation SOP of the *DNA Procedures Manual*. The sex typing results from the amelogenin locus are not included in kinship index calculations.

⁴ Not all References will add value to the calculation. For example, if both parents are available, adding a sibling to the calculation does not change the final result. Likewise, if the father is available, the paternal grandparents are not needed. Only the References that contain additional genetic information not otherwise represented need to be used in the calculation.

KIT CONVERSION WORKSHEET

- 1) Using the drop-down menus, arrange the loci in the order of the commercial multiplex employed.
- 2) Click on the respective button to insert the Kin CALc conversion into the Profiles worksheet.

Kin CALc 5.0.10 FBI

Commercial Multiplex		Kin CALc	
Format	Item #	Format	Item #
D3S1358	Allele 1	CSF1PO	Allele 1
	Allele 2		Allele 2
vWA	Allele 1	D10S1248	Allele 1
	Allele 2		Allele 2
D16S539	Allele 1	D12S391	Allele 1
	Allele 2		Allele 2
CSF1PO	Allele 1	D13S317	Allele 1
	Allele 2		Allele 2
TPOX	Allele 1	D16S539	Allele 1
	Allele 2		Allele 2
D8S1179	Allele 1	D18S51	Allele 1
	Allele 2		Allele 2
D21S11	Allele 1	D19S433	Allele 1
	Allele 2		Allele 2
D18S51	Allele 1	D1S1656	Allele 1
	Allele 2		Allele 2
D2S441	Allele 1	D21S11	Allele 1
	Allele 2		Allele 2
D19S433	Allele 1	D22S1045	Allele 1
	Allele 2		Allele 2
TH01	Allele 1	D2S1338	Allele 1
	Allele 2		Allele 2
FGA	Allele 1	D2S441	Allele 1
	Allele 2		Allele 2
D22S1045	Allele 1	D3S1358	Allele 1
	Allele 2		Allele 2
D5S818	Allele 1	D5S818	Allele 1
	Allele 2		Allele 2
D13S317	Allele 1	D6S1043	Allele 1
	Allele 2		Allele 2
D7S820	Allele 1	D7S820	Allele 1
	Allele 2		Allele 2
SE33	Allele 1	D8S1179	Allele 1
	Allele 2		Allele 2
D10S1248	Allele 1	F13A01	Allele 1
	Allele 2		Allele 2
D1S1656	Allele 1	F13B	Allele 1
	Allele 2		Allele 2
D12S391	Allele 1	FESFPS	Allele 1
	Allele 2		Allele 2
D2S1338	Allele 1	FGA	Allele 1
	Allele 2		Allele 2

Insert Test Profile

Insert AR1 Profile

Insert AR2 Profile

Insert AR3 Profile

Insert AR4 Profile

Insert AR5 Profile

Insert AR6 Profile

Insert AR7 Profile

Insert AR8 Profile

Insert AR9 Profile

Insert AR10 Profile

Clear Data

To Profiles

To Intro

To Pedigree

To Custom Pedigree

To KI

Figure 1 – Entering profiles in the “Kit Conversion” tab

4.1.2.3.2 Option 2: Import txt file from GeneMapper® ID-X (GMIDX)

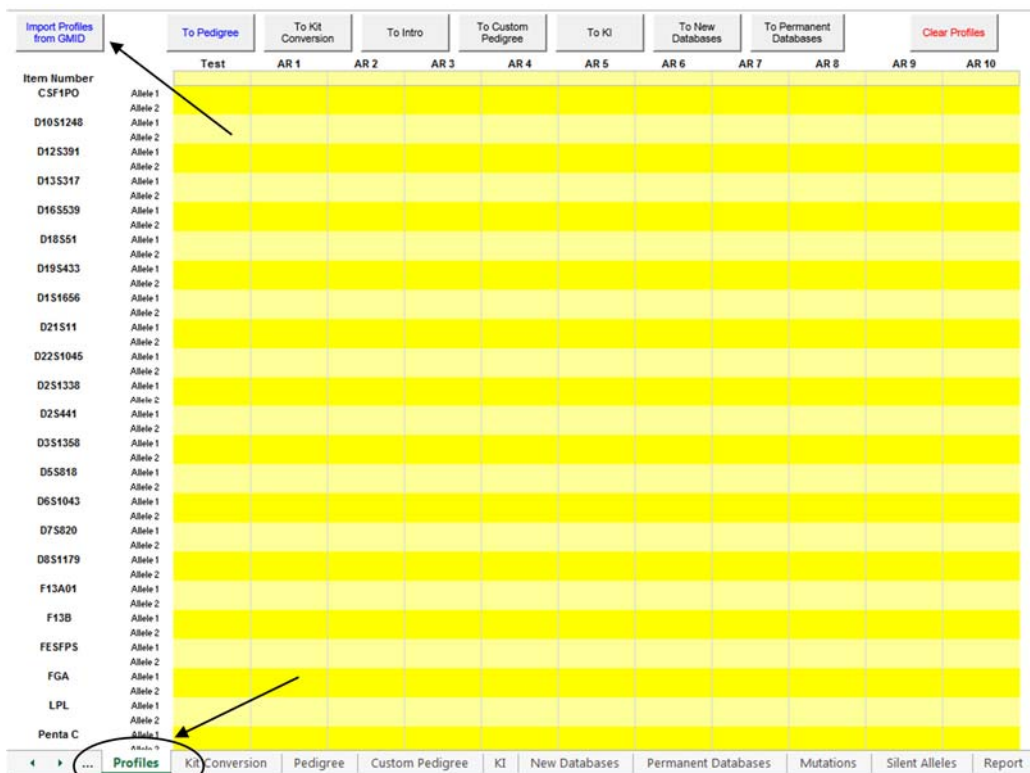
4.1.2.3.2.1 In GeneMapper® ID-X (GMIDX), highlight the sample(s) to be imported into KIn CALc and click on the “Display Plots” icon.

Redacted

4.1.2.3.2.2 From the plots, select the “Sizing Table” icon. Go to “File” – “Export Table”. Save the generated .txt file.

Redacted

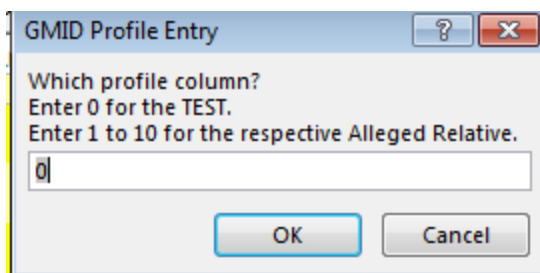
4.1.2.3.2.3 Open the KIn CALc software and navigate to the “Profiles” tab. Click on the “Import Profiles from GMID” button located on the upper left hand corner of the screen.



4.1.2.3.2.4 Locate exported table and click “Open”. From the “Import Profile from an Exported Table” box, select the profile (one at a time) to be imported into the software and click “OK”. Ensure that the displayed sample name is correct and click “OK”. If the sample name is incorrect, the user can make the required changes in the appropriate field before continuing to the next step.

Redacted

enter sequential numbers from “1-10” for each of the samples until all of the profiles have been entered.



4.1.2.3.2.6 Repeat the previous steps until all samples needed to establish the pedigree have been entered into the software tool.

4.1.2.3.2.7 Once all references are entered, click the “To Pedigree” or “To Custom Pedigree” button as appropriate or manually navigate to the appropriate tab.

4.1.2.4 Establishing Alleged Relationship(s) on the Pedigree

Navigate to the “Pedigree” or “Custom Pedigree” tab at the bottom of the screen, as appropriate. The custom pedigree tab should be used in situations that cannot be evaluated using the standard pedigree tab (see 4.1.2.4.2).

4.1.2.4.1 Pedigree Tab for Standard Pedigrees

4.1.2.4.1.1 In the pedigree screen, the Test will always be pre-selected. Select the box(es) corresponding to the reference sample(s) relationship(s) to the Test. For example, if item 1 is the alleged mother of the Test, select the box in the pedigree that corresponds to the mother. If item 2 is the alleged full-sibling of the Test, select a box in the pedigree that corresponds to a full-sibling. See Figure 2. For paternity cases, the alleged parent will always be the ‘Test’ and the known parent and child will be assigned the boxes corresponding to ‘Test Mate’ and ‘Test-Test Mate-Child’, respectively. See Figure 3.

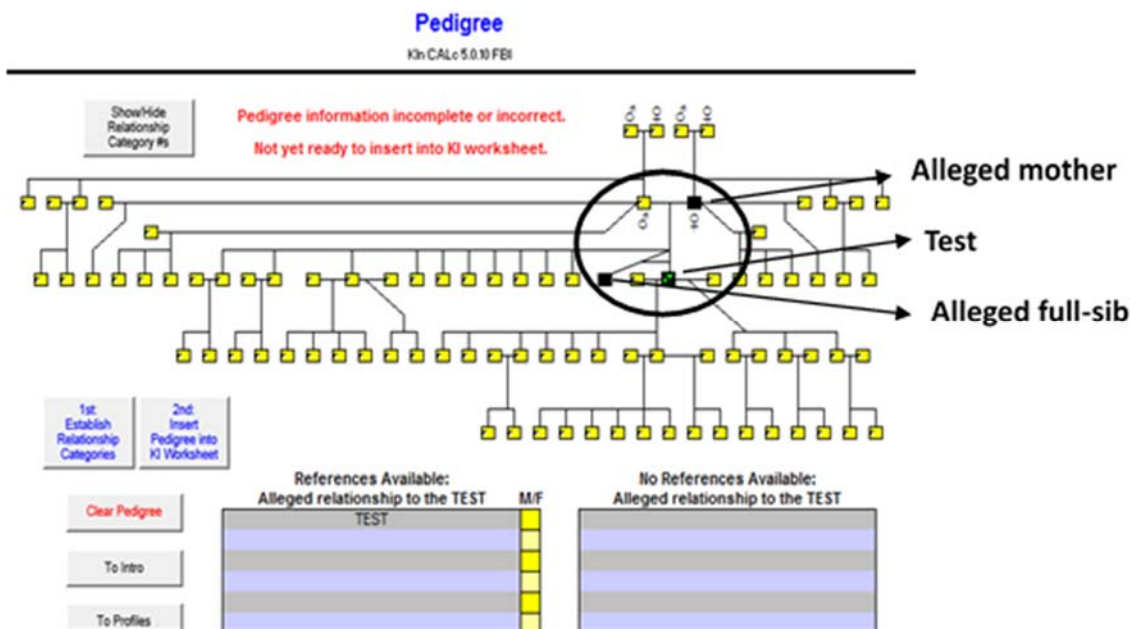


Figure 2 – The “Pedigree” tab

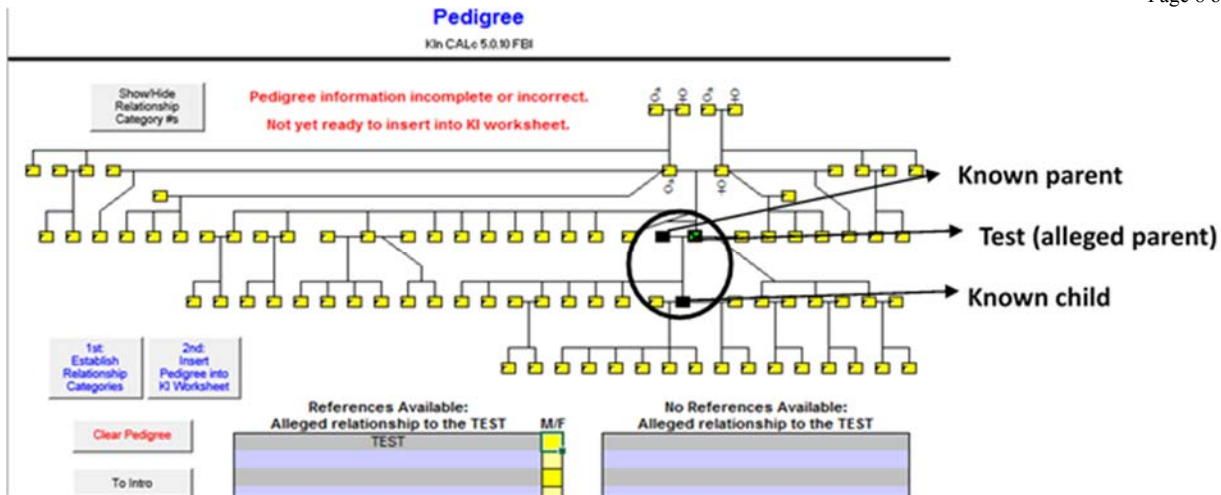


Figure 3 – The “Pedigree” tab for Paternity Analysis

4.1.2.4.1.2 Click on the “1st: Establish Relationship Categories” button on the left side of the screen. This action will populate the “References Available” box with the relatives selected in the pedigree. The “No References Available” Box will be auto-populated with those relatives for which information is not entered but whose information would have been required to establish the genotypes of the available individuals. For example, to correctly infer the genotype of the Test and full-sibling, both parents’ genotypes are required. If the genotype for the father is not entered, the software will generate his potential genotypes in order to perform the calculation; therefore, “father” will be auto-populated in the “No References Available box.” See Figure 4.

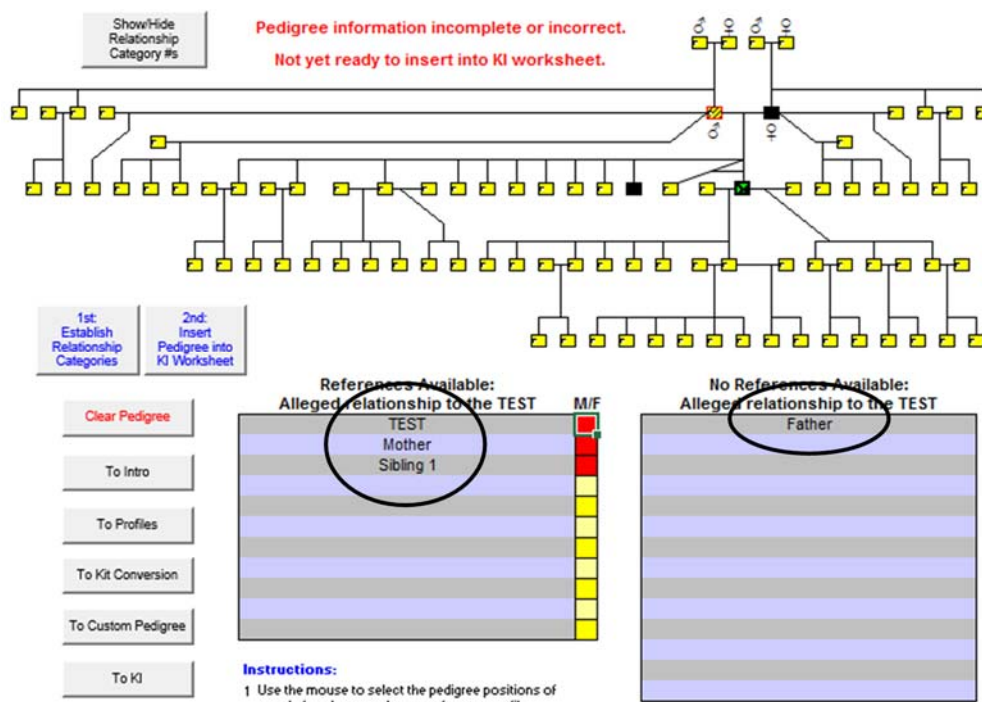


Figure 4 – Establishing Relationship Categories in the “Pedigree” tab

4.1.2.4.1.3 Verify that the relationships described in the References available box correspond to the actual References available. If not, go back to the pedigree and deselect the incorrect box in the pedigree, then select the correct pedigree box and click on the “1st: Establish Relationship Categories” button again. The “References Available” and “No References Available” boxes will be updated with the new information.

4.1.2.4.1.4 Enter the gender of the Test and References by typing the letter “M” for male and “F” for female in the red box next to the relative listed in the “Reference available” box. The boxes will change to yellow once the information is entered and will all turn green once the gender for all samples are entered. The message in red at the top of the screen will change to green to indicate that all the information needed to perform the kinship index calculation has been completed and the user can proceed to the calculation screen. See Figure 5.

Figure 5 shows the 'Pedigree' tab interface. At the top, a message box states 'Pedigree complete. Ready to insert into KI worksheet.' Below this is a pedigree chart. To the left of the chart are two buttons: '1st: Establish Relationship Categories' and '2nd: Insert Pedigree into KI Worksheet'. Below the chart is a 'References Available' table with columns 'Alleged relationship to the TEST' and 'M/F'. The table lists 'TEST', 'Mother', and 'Sibling 1'. The 'TEST' row has a green box with an 'X' in the 'M' column. To the right of the 'References Available' table is a 'No References Available' table with the column 'Alleged relationship to the TEST' and the entry 'Father'. Below the tables are instructions and a note.

Instructions:

- 1 Use the mouse to select the pedigree positions of people for whom you have a reference profile.
- 2 Click on "Establish Relationship Categories".
- 3 Enter the known gender of the people with references, regardless of whether or not there are gender identifying DNA results.
- 4 Click on "Insert Pedigree into KI Worksheet".

Note:

- The green box with an X in it is the position of the TEST person (e.g., the possible remains of a missing person, or the alleged father in a paternity.) This is the person who is in question, and whose inclusion in the pedigree

Figure 5 – Entering gender in the “Pedigree” tab

4.1.2.4.2 Custom Pedigree Tab for Non-Standard Pedigrees

Drawing the pedigree(s) can aid in the use of the custom pedigree function. The custom pedigree tab may be used in two situations. Use of the custom pedigree tab to assess any scenario besides the two described below may only be conducted with TL approval.

(A) To assess scenarios for which:

- mutations must be allowed for the pedigree to be true,
- the No References Available box is populated, and

- relationships other than parents or grandparents are populated in the “No References Available” box because mutation rates are gender specific.

In this scenario, the alternative hypothesis is unrelatedness. Go to the Custom Pedigree tab and set the “Manual alternate pedigree?” to “NO”, which prompts the software to automatically calculate the default denominator (i.e., unrelatedness). Enter the appropriate information in the “References Available” and “References Not Available” boxes, only using the “Primary Pedigree” area. Assign numbers 1-10 to samples for which genetic information is available, and 20-33 for any samples without genetic information but which are necessary to assess the relationship(s) in question. The “Test” sample will always be represented by number 17.

(B) To assess scenarios for which the alternate hypothesis is **not** unrelatedness: Go to the Custom Pedigree tab and set the “Manual Alternate Pedigree?” to “YES”, which allows for the alternate hypothesis to be defined. Using the “References Available” and “References Not Available” boxes, fill out the information corresponding to the Numerator in the “Primary Pedigree” area and the Denominator in the “Manual/Alternate Pedigree” area. Assign numbers 1-10 to samples for which genetic information is available, and 20-33 for any samples without genetic information but which are necessary to assess the relationship(s) in question. The “Test” sample will always be represented by number 17. The Primary Pedigree and Manual Alternate Pedigrees are independent; therefore, an individual represented by “1” in the Primary Pedigree does not need to be “1” in the Manual Alternate Pedigree. **When entering the information in the References boxes, always start from the most distant relatives available.**

- For example, are items 1 and 2 more likely from $\frac{3}{4}$ siblings (the fathers of the alleged siblings are full brothers, and the alleged siblings have the same mother) or from $\frac{1}{2}$ siblings (the fathers of the alleged siblings are unrelated, and the alleged siblings have the same mother)? Figure 7 shows the pedigrees generated for each of the two hypotheses. Figure 8 shows the custom pedigree tab for this example.

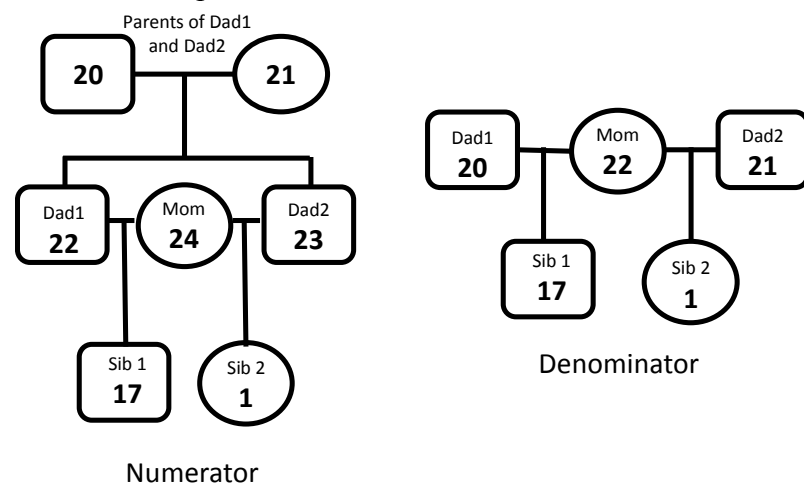


Figure 7 – Numerator and Denominator Hypothesis Pedigrees

CUSTOM PEDIGREES Kin CALc 5.0.10 FBI [See below for instructions.](#)

Pedigrees that will not fit into the structure found on the Pedigree worksheet may be entered here.
Note: The report for KIs calculated using a Custom Pedigree will not have a graphical component.

Manual alternate pedigree? ☒ **Yes** OK to transfer to KI? ☒ **TRUE**

Note: Entries in the second pedigree will not be used unless "Yes" is selected.

Monozygotic twin category:

Note: Monozygotic twin must be the last reference entered. Do not enter parents for this person.

[Insert Pedigree\(s\) into KI Worksheet](#)

References Available

Pedigree Member's Category	Gender (M/F)	Biological Father's Category	Biological Mother's Category
17	M	22	24
1	F	23	24
2			
3			
4			
5			
6			
7			
8			
9			
10			

Note: Do not skip rows when entering the pedigree(s).

References Not Available

Pedigree Member's Category	Gender (M/F)	Biological Father's Category	Biological Mother's Category
20	M		
21	F		
22	M	20	21
23	M	20	21
24	F		
25			
26			
27			
28			
29			
30			
31			
32			
33			

References Available

Pedigree Member's Category	Gender (M/F)	Biological Father's Category	Biological Mother's Category
17	M	20	22
1	F	21	22
2			
3			
4			
5			
6			
7			
8			
9			
10			

References Not Available

Pedigree Member's Category	Gender (M/F)	Biological Father's Category	Biological Mother's Category
20	M		
21	M		
22	F		
23			
24			
25			
26			
27			
28			
29			
30			
31			
32			
33			

Figure 8 – Custom Pedigree Tab

4.1.2.4.3 Performing the Kinship Index (KI) (or Paternity Index [PI]) Calculation

4.1.2.4.3.1 From either the Pedigree tab or the Custom Pedigree tab, click “2nd: Insert Pedigree into KI Worksheet.” This action will take the user to the calculator screen on the “KI” tab.

4.1.2.4.3.2 Go to the “Item #” column and select the appropriate sample identifier label from the drop-down menu for each of the samples. As the sample identifiers are selected, the software will populate loci and allele columns with the DNA data entered by the user in the “Profile ID” tab. See Figure 9.

The screenshot shows the 'KI' tab in a software interface. At the top, there is a pedigree chart with a grid of cells representing relationships. Below the pedigree, there is a table of sample identifiers (CYP19D, D1S10, etc.) with columns for 'Num', 'Den', 'KI', and 'Onset'. A red circle highlights the 'Relationship to the test person' dropdown menu. On the right side, there is a 'PROGRESS MARKERS' section with a table showing the progress of calculations for different sample identifiers. The table has columns for 'Sample ID', 'Status', 'Turn this on?', and 'Turn this off?'. The 'Status' column shows 'No' for all samples. The 'Turn this on?' and 'Turn this off?' columns are empty. Below the table, there is a note: 'Note: % Complete is only displayed when the Allele approach is 0.' At the bottom, there is a 'Select Database:' section with radio buttons for 'African', 'Caucasian', and 'SW Hispanic'. The 'African' button is selected. Below this, there is a 'General allele probability settings' section with a 'Show' button.

Figure 9 – Associating sample identifiers with the pedigree in the “KI” tab

4.1.2.4.3.3 From the drop-down menus at the bottom of the screen, the user must select the population frequency databases with which calculations will be performed. Kinship indices are calculated using four United States population groups (i.e., African-American, Caucasian, Southwestern Hispanic, and Southeastern Hispanic). Additional kinship indices are calculated when samples potentially originate from Native American populations (i.e., Apache, Navajo, and Minnesota Native American), or Caribbean populations (i.e., Trinidadian).^{5,6} See Figure 10. Note that selecting a “blank” database (e.g., when Southeastern Hispanic is calculated) will default the column to a previously used database. Select “No” for “Report this?” under the combined KI so that the value is not included on the report. The maximum number of populations per KIn CALc report is three. Generally, the African-American, Caucasian, and Southwestern Hispanic databases are selected first.

⁵ The use of the Native American or Caribbean population databases is generally based on the geographic location of the requesting agency or the population with which the known references in the pedigree associate themselves. With TL approval, other population databases may be similarly used.

⁶ The allele frequency distributions for the African American, Caucasian, Southeastern Hispanic, Southwestern Hispanic, Apache, Navajo, Trinidadian, Chamorro, and Filipino populations are published in: Moretti TR, Moreno LI, Smerick JB, Pignone ML, Hizon R, Buckleton JS, Bright J-A, Onorato AJ. Population data on the expanded CODIS core STR loci for eleven populations of significance for forensic DNA analyses in the United States. *Forensic Science International: Genetics* (2016) 25: 175-181. The allele frequency distributions for the Minnesota Native American population are found in the appropriate *DNA Procedures Manual*. Other sources of allele frequency distributions must be approved by the TL.

Figure 10 – Selecting population databases in the “KI” tab

4.1.2.4.3.4 By default, the “Mutations” box is set to “0,” which means that the software will not allow for mutations in the calculation. Based on comparisons of DNA typing results of the Test and Reference samples, if inconsistencies in the typing results indicate that a mutation might have occurred (at three loci or less), the Examiner should click on the “Change Mutation Approach” button on the lower right side of the screen and select “1” for the mutation approach.^{7,8}

4.1.2.4.3.5 The gender of all samples in the pedigree must be defined for calculations that include mutations.⁹ If a mutation is necessary for the pedigree to be true, the examiner should ensure that the No References Available list in the Pedigree tab are only those with pre-determined genders in the pedigree (i.e., parents or grandparents of the TEST). If they are not, the pedigree should be constructed in the custom pedigree tab so that the genders of all individuals for both the References Available and No References Available can be defined before calculating the KI.

4.1.2.4.3.6 Click on the “KI” button; this action will populate the chosen population columns with the values corresponding to the numerator, denominator and likelihood ratio result for each of the loci for which information was entered. It will also compute the “Combined KI” (product of individual locus KIs) for each of the populations. If the kinship index is zero, the pedigree cannot be true without allowing mutations. The locus(i) requiring a mutation allowance can be identified by looking at the “Num” (Numerator) and “Den” (Denominator) column for each locus. Any locus with a value of “0” means mutations must be allowed for the pedigree to be true. Refer to 4.1.2.4.3.4 to change the mutation approach.

⁷ This approach was described by Ayres (2000) and is applied to all loci in the profile regardless of which locus requires the allowance for mutation.

⁸ When the DNA typing results of one of multiple relatives is not consistent with the pedigree in the manner described by the contributor, the Examiner may perform two calculations: one with all of the relatives, and one with the relative that does not fit removed. Both KIn CALc reports will be maintained, and the Examiner may report the results with fewer relatives.

⁹ To perform the mutation calculations, the software utilizes a gender specific rate of mutation for each locus.

4.1.2.4.3.7 If loci should be omitted from the KI calculation because of linkage, select “Yes” in the “Omit?” column next to the locus for each population. Most commonly, D5S818 and vWA are omitted from pedigrees that are not simple paternity or simple reverse paternity. See 4.1.2.2 for additional information to determine which loci should be omitted.

4.1.2.4.3.8 Enter the lab number, Examiner name and/or symbols and date in the corresponding boxes.

4.1.2.4.4 Generating the Report

4.1.2.4.4.1 Navigate to the appropriate report tab. For scenarios in which custom pedigrees were used, the reports will be found on the “Report Cust Ped 1” tab if the manual alternate pedigree setting was set to “No” and the “Report Cust Ped 2” if it was set to “Yes”.

For paternity cases, click on the “Paternity” button at the bottom of the report sheet. The reported combined KI is equivalent to the combined paternity index (PI). Additionally, the probability of paternity calculation and an explanatory statement for the prior probability applied to the calculation will appear. See Figure 11.

Return to KI

To Report Page 2

Print Report

Print This Page Only

Item #	Legal relationship to the TEST individual
	TEST

Combined KI

AFR

Caucasian

SW Hispanic

Probability of Paternity

Probability of paternity was calculated assuming a prior probability of 50%. Prior probability refers to the initial odds of the alleged father's paternity before genetic testing is performed.

Databases: References:

AfAm FSI Genetics 25 (2016) 175-181

Caucasian FSI Genetics 25 (2016) 175-181

SW Hispanic FSI Genetics 25 (2016) 175-181

Allele probability: $1/2N$

Minimum probability for rare alleles: $5/2N$

Theta set to 0. Mutations were not allowed. Silent alleles were not considered.

PATERNITY

Add KI/Pool

Figure 11 – Viewing the “Report” tab for Paternity Analysis

4.1.2.4.4.2 Verify that the displayed information is correct (Lab number, Examiner, date, relationships and profiles). If incorrect information is noted, the user can navigate back to previous tabs to correct the information. The alleles can be corrected in the “Profiles ID” sheet, the pedigree can be corrected in the “Pedigree” or “Custom Pedigree” sheet, and the sample identifiers can be corrected in the “KI” sheet. The user can navigate to these sheets either by selecting the corresponding tabs at the bottom of the workbook or by using the “Return to KI” button and then selecting the “To Profiles ID” or “To Pedigree” buttons. Once changes are made, follow the procedure from the corresponding step forward so that the correct calculation is obtained.

4.1.2.4.4.3 All profiles used to generate the calculations are available on the second page of the report. This second page can be accessed by clicking on “To Report Page 2” or navigating to the tab with the same name.

4.1.2.4.4.4 Click on the “Print Report” button to print both pages of the report.

4.1.2.4.4.5 Once the KIn CALc report is generated, the user may go to the KI tab to calculate the statistic(s) for additional population(s), as appropriate.

4.1.2.4.4.6 To perform calculations for another pedigree (e.g., a different case), click on the “Return to KI” button from the report view. On the KI calculator screen, click on the “Clear Data” button. On the Kit Conversion screen, click on “Clear Data.” The software is now ready for a new analysis.

4.1.2.4.4.7 Once all reports have been printed, the user can proceed to close the software **without saving changes.**

5 Interpretation of Kinship Analysis Results

The weight of the statistical value varies depending on the available reference samples and their relationship to the Test. If first degree relatives are available (e.g., parents, offspring, full-siblings), the probability of obtaining a high KI is increased as compared to when second degree relatives (e.g., half-siblings, grandparents, uncle/aunt) or third degree relatives (e.g., cousins) are used for comparison. Generally, the highest KI's are expected for the pedigrees listed in Section A of Table 1.¹⁰ The pedigrees listed under Sections B and C are less informative.

Whenever possible, the contributor should be directed to collect samples that have a potential to give the greatest KI. Testing additional relatives, if available, can further refine the relatedness of the individuals in question, especially in instances where the combined KI is low.¹¹

¹⁰ Table 1 was adapted from Ge et. al., 2011.

¹¹ Lineage markers (i.e., mitochondrial DNA or Y-STR typing) can also aid in establishing relatedness.

Available known references
SECTION A
3 children + spouse
4 children
Both parents
2 spouses + 2 children (1 each)
2 children + spouse
3 children
1 parent + 3 full-siblings
1 child + 1 parent + spouse
Spouse + 1 child + 1 child w/2 nd spouse
4 full siblings
1 full-sibling + 1 child + spouse
1 parent + 2 full-siblings
3 full-siblings
1 child + 1 parent
2 children
1 full sibling + 1 child
1 full sibling + 1 parent
1 child + spouse
2 full siblings
1 half sibling + 1 parent (not the parent of the half sibling)
1 uncle + 1 parent (they are not related)
1 grandchildren + 1 child (they are uncle/nephew)
1 parent OR 1 child
SECTION B
1 half sibling + 1 full sibling
1 full sibling
SECTION C
2 uncles (they are not related)
2 grandchildren (who are cousins)
2 half siblings (2 halvesibs are also halvesibs)
2 half siblings (2 halvesibs are fullsibs)
2 grandchildren (who are fullsibs)
2 uncles (who are fullsibs)
1 grandparent OR 1 grandchild
1 uncle OR 1 nephew
1 half sibling
2 cousins (they are also cousins)
2 cousins (they are full siblings)
1 cousin

Table 1

5.1 Reporting Procedures

5.1.1 A statement should be included to define the relationships of the submitted reference samples to each other and, if appropriate, the named missing person. This information should be reported generally as follows:

Redacted is identified by the incoming communication from the contributor as the biological mother of the missing person,

are identified by the incoming communication from the contributor as the biological mother and brother, respectively, of the missing person,
”

is identified by the incoming communication from the contributor as the biological mother of ”

5.1.2 If Y-STR or mitochondrial DNA testing has excluded the putative relationship, no kinship analysis will be conducted for the specimens in question. A statement explaining the reason no comparisons were conducted will be included generally as follows:

“The request for the nuclear DNA comparison of item 1 to Redacted was not performed due to the exclusionary results of the Y-STR comparisons.”

*“The request for the nuclear DNA comparison of item 1 to specimen
was not performed due to the exclusionary results of the mitochondrial DNA comparison provided in the FBI Laboratory report dated December 1, 2000.”*

“The request for the nuclear DNA comparison of item 3 to was not performed due to the exclusionary results of the mitochondrial DNA comparison provided in the State Police Laboratory report dated December 1, 2000, for .”

5.1.3 Unrelated Specimens: Exclusions

5.1.3.1 The comparison of individual DNA profiles in relationships that have an expected pattern of inheritance of alleles (e.g., parent-offspring) may be deemed an exclusion when the expected pattern of allele transmission is not observed. The ability to exclude is limited to those instances in which the STR typing results of the References are able to define or partially define the potential alleles for the Test at a given locus(i). Generally, the ability to exclude is limited to: (1) parent-offspring, (2) multiple siblings, (3) both maternal grandparents, or (4) both paternal grandparents. Combinations of these relatives with additional first and second degree relatives may also allow the Examiner to exclude. Other relative scenarios may also result in an exclusion, but they require numerous relatives (e.g., multiple maternal or paternal aunts/uncles).

5.1.3.2 An exclusion may be declared when the DNA typing results do not fit the proposed

relationships at four or more corresponding loci. At each locus that violates the pedigree, the software will give a KI of 0 or less for that locus and will shade the denominator box(es) in red or the result box(es) in purple. This shading should prompt the Examiner to determine if a mutation approach should be employed. If only one, two, or three loci are shaded, the Examiner should apply the mutation approach. An exclusionary conclusion should be reported generally as follows:

“Based on the STR typing results, item 1 is excluded as being from a biological offspring of _____; therefore, item 1 could not have originated from _____.”

“Based on the STR typing results, _____ is excluded as being the biological father of _____.”

“Based on the STR typing results, item 1 is excluded as a being from the biological father of _____. Therefore, item 1 could not have originated from _____.”

“Based on the STR typing results, item 1 is excluded as being from a sister of _____. Therefore, item 1 could not have originated from _____.”

“Based on the STR typing results, item 1 is excluded as a being from a potential grandchild of _____ and _____. Therefore, item 1 could not have originated from _____.”

5.1.4 Inconclusive Results

5.1.4.1 A DNA profile is inconclusive for statistical purposes if, at all loci for which results were obtained, only one allele is detected and it is below the stochastic threshold (ST).¹²

5.1.4.2 If a DNA profile is inconclusive at some loci, but is conclusive at one or more loci, the conclusive loci may be used for statistical calculations as described. All loci should be considered when assessing relatedness.

5.1.4.3 Any profile with 3 or fewer inconclusive loci, and no conclusive loci, is unsuitable for comparisons because 4 loci are needed to exclude from a pedigree.

5.1.4.4 If enough pedigree information is available and at least 4 loci exclude relatedness, an inconclusive DNA profile may be used for an exclusion. Pedigrees that may allow for exclusions include both parents, a spouse and offspring, or multiple siblings.

5.1.4.5 If the pedigree information is too limited to exclude relatedness (e.g., only a single

¹² Refer to the appropriate interpretation SOP of the *DNA Procedures Manual* for determination of inconclusive profiles.

relative is available for comparison), an inconclusive DNA profile cannot be used for comparisons.

5.1.4.6 A statement that describes the inconclusive result and the limitations of the comparisons should be included generally as follows:

“The STR typing results obtained for item 1 are not suitable to determine relatedness; however, they may be utilized for exclusionary purposes. Based on the STR typing results, item 1 is excluded as being from an offspring of _____; therefore, item 1 could not have originated from _____.”*

“The STR typing results obtained for item 1 are not suitable to determine relatedness; however, they may be utilized for exclusionary purposes. Based on the STR typing results, no comparison information for item 1 can be provided for _____.”*

“The limited STR typing results obtained for item 1 are not suitable to determine relatedness. Therefore, no comparisons were made to _____.”*

Each with the following explanatory endnote:

*“*STR typing results are deemed not suitable to determine relatedness (i.e., inconclusive) when the potential exists that not all of the genetic information in a biological sample has been detected. For STR typing results to be used to determine relatedness, sufficient DNA quality and/or quantity is necessary, and will depend on the relative samples submitted.”*

5.1.5 Reporting Combined Kinship Indices

5.1.5.1 Combined kinship indices¹³ are calculated using four general United States population groups (i.e., African-American, Caucasian, Southwestern Hispanic, and Southeastern Hispanic). Additional kinship indices may be reported for specimens that potentially originated from Native American populations (i.e., Apache, Navajo, and Minnesota Native American) or Caribbean populations (i.e., Trinidadian).¹⁴ The lowest combined KI should be reported generally as follows:

“Based on the STR typing results and the comparisons of item 1 to _____, the combined kinship index is approximately 150 million.”*

¹³ All kinship indices are reported rounded to two significant figures as provided by KIn CALc, or, if between 1 and 10, truncated to 1 significant figure. For example, 13,423 is rounded to 13,000; 54,784,652 is rounded to 55,000,000, or 55 million; 3,751,768,135 is rounded to 3,800,000,000, or 3.8 billion.

¹⁴ The use of Native American or Caribbean population databases is generally based on the geographic location of the requesting agency or the population associated with the pedigree. With TL approval, other population databases may be similarly used.

with the following associated explanatory endnote:

*“*Not all loci at which amplification is attempted will yield interpretable results; a statistical estimate (combined kinship index) has been based on loci with conclusive typing results. Calculations were performed using the African American, Caucasian, Southeastern Hispanic, and Southwestern Hispanic populations. The lowest combined kinship index calculated from these populations is reported.”¹⁵*

5.1.5.2 The combined paternity index (PI) is a specialized combined kinship index. It is calculated for the four general United States population groups (i.e., African-American, Caucasian, Southwestern Hispanic, and Southeastern Hispanic). Additional kinship indices may be reported for specimens that potentially originated from Native American populations (i.e., Apache, Navajo, and Minnesota Native American) or Caribbean populations (i.e., Trinidadian). The lowest combined PI and the probability of paternity should be reported generally as follows (where Redacted is the alleged father, Redacted is the known mother, and Redacted is the child):

“Based on the STR typing results and the comparisons of Redacted, the combined paternity index is approximately 23,000 **”*

with the following explanatory endnotes, which include the probability of paternity:

*“*Not all loci at which amplification is attempted will yield interpretable results; a statistical estimate (combined paternity index) has been based on loci with conclusive typing results. Calculations were performed using the African American, Caucasian, Southeastern Hispanic, and Southwestern Hispanic populations. The lowest combined paternity index calculated from these populations is reported.”*

***The corresponding probability of paternity is 99.995%. The probability of paternity is expressed as a percentage that incorporates the combined paternity index and a 50% prior probability that the tested man is the biological father of the child.”*

5.1.5.3 Section A Pedigrees

Pedigrees with the most value for kinship analysis consist of individual or multiple first degree relatives,¹⁶ or a first degree relative along with a second degree relative.¹⁷ These pedigrees, which also include pedigrees for paternity analysis, are captured in Section A of Table 1.

5.1.5.3.1 If the reported combined kinship index is greater than one, an additional clarifying

¹⁵ This endnote is only appropriate for analyses where the alternate hypothesis is unrelatedness. This endnote should be modified as appropriate to address relationships assessed by multi-category pairing or custom pedigrees.

¹⁶ A first degree relative is a direct descendant, predecessor, or full sibling of the person in question, i.e., a parent, child, or full sibling. Additionally, though spouse is not a biological relative, it is important to the evaluation if children are available for typing.

¹⁷ A second degree relative is a direct descendant, predecessor, or full sibling of a first order relative, i.e., a grandparent, grandchild, or full sibling of the parent of the person in question.

statement will be added generally as follows:

“Therefore, the profile obtained from item 1 is approximately 150 million times more likely if item 1 is from the child of Redacted than if item 1 is from someone unrelated to these individuals.”

“Therefore, the profile obtained from item 1 is approximately 150 million times more likely if item 1 is from the sister of Redacted than if item 1 is from someone unrelated to these individuals.”

“The profile for Redacted [child] is approximately 280 million times more likely if Redacted [known parent] and Redacted [alleged parent] are the parents than if Redacted [known parent] and an unrelated randomly selected man are the parents.”

5.1.5.3.2 A statement summarizing the strength of the evidence should follow. See Table 2 for a summary of reporting language for Section A pedigrees.

Combined KI of	Reported as
100,000 or greater	Strong evidence
1,000 to 99,999	Evidence
100 to 999	Cannot be excluded
1 to 99	Insufficient support to conclude relatedness
0 to 1	Unlikely

Table 2 - Reporting Language for Section A Pedigrees

5.1.5.3.3 Report Wording Examples for Section A Pedigrees

The qualitative equivalent of the combined KI or PI is based on the magnitude of the reported LR. These conclusions should be reported generally as follows:

“These results provide strong evidence that item 1 originated from Redacted .”*

“These results provide strong evidence that Redacted is the biological father of Redacted .”*

“These results provide evidence that item 1 originated from Redacted .”*

“These results provide evidence that Redacted is the biological father of Redacted .”*

“Therefore, item 1 cannot be excluded as having originated from Redacted .”*

“Therefore, Redacted cannot be excluded as the biological father of Redacted .”*

“These results provide insufficient support to conclude that item 1 originated from Redacted .”*

“These results provide insufficient support to conclude that is the biological father of .”*

“Therefore, it is unlikely that item 1 originated from .”*

“Therefore, it is unlikely that is the biological father of .”*

with the following associated explanatory endnote:

*“*These combined kinship/paternity index (KI/PI) ranges provide the following support for the conclusion:*

<u>KI/PI</u>	<u>Qualitative Equivalent</u>
$\geq 100,000$	<i>strong evidence of relatedness</i>
1,000 to 99,999	<i>evidence of relatedness</i>
100 to 999	<i>cannot be excluded as related</i>
1 to 99	<i>insufficient support to conclude relatedness</i>
0 to 1	<i>unlikely to be related”</i>

5.1.5.4 Section B Pedigrees

Pedigrees consisting of a single full sibling or a full sibling and a half sibling are sometimes useful in establishing kinship. However, comparison of true relatives in this category will occasionally result in KIs between zero and one. Additionally, there is a potential for fortuitous associations. Therefore, the report should include a request for additional, informative, relative samples from the contributor. These two pedigree types are captured in Section B of Table 1.

5.1.5.4.1 If the reported combined kinship index is greater than one, an additional clarifying statement should generally as follows:

“Therefore, the profile obtained from item 1 is approximately 4,500 times more likely if item 1 is from the brother of than if item 1 is from someone unrelated to this individual.”

“Therefore, the profile obtained from item 1 is approximately 4,500 times more likely if item 1 is from the brother and half-brother, respectively, of than if item 1 is from someone unrelated to these individuals.”

5.1.5.4.2 A statement summarizing the strength of the evidence should follow. See Table 3 for a summary of reporting language for Section B pedigrees.

Combined KI of	Reported as
100,000 or greater	Strong evidence
1,000 to 99,999	Evidence
100 to 999	Cannot be excluded
0 to 99	Insufficient support to conclude relatedness

Table 3 - Reporting Language for Section B Pedigrees

5.1.5.4.3 Report Wording Examples for Section B Pedigrees

The qualitative equivalent of the combined KI is based on the magnitude of the reported LR. These conclusions should be reported generally as follows:

“These results provide strong evidence that item 1 originated from Redacted ; however, further testing from at least one additional relative (preferably a parent, child, or full sibling) is recommended to support this conclusion.”*

“These results provide evidence that item 1 originated from Redacted ; however, further testing from at least one additional relative (preferably a parent, child, or full sibling) is recommended to support this conclusion.”*

“Therefore, item 1 cannot be excluded as having originated from Redacted ; however, further testing from at least one additional relative (preferably a parent, child, or full sibling) is recommended to support this conclusion.”*

“These results provide insufficient support to conclude that item 1 originated from Redacted ; however, further testing from at least one additional relative (preferably a parent, child, or full sibling) is recommended.”*

with the following associated explanatory endnote:

*“*These combined kinship index (KI) ranges provide the following support for the conclusion:*

<u>KI</u>	<u>Qualitative Equivalent</u>
$\geq 100,000$	<i>strong evidence of relatedness</i>
1,000 to 99,999	<i>evidence of relatedness</i>
100 to 999	<i>cannot be excluded as related</i>
0 to 99	<i>insufficient support to conclude relatedness”</i>

5.1.5.5 Section C Pedigrees

Pedigrees consisting of one or two second degree relatives¹⁸ or third degree relatives¹⁹ are at times useful in establishing kinship. However, comparison of true relatives in this category may result in low KIs and KIs less than one. Additionally, as with Section B pedigrees, there is a potential for fortuitous associations. These pedigree types are captured in Section C of Table 1. Because of the limitations in the analysis of these pedigrees, there are only two categories of reporting. Additionally, the report should include a request for additional, informative, relative samples from the contributor.

5.1.5.5.1 A statement explaining the limited value of the relationships should be added to the paragraph identifying the relatives submitted for analysis, generally as follows:

Redacted is identified by the incoming communication from the contributor as the maternal uncle of the missing person, Redacted. It is noted that comparisons to maternal uncles have limited value because second degree relatives are expected to share limited genetic information by descent."

Redacted are identified by the incoming communication from the contributor as the cousins of the missing person, Redacted. It is noted that comparisons to cousins have limited value because third degree relatives are expected to share limited genetic information by descent."

5.1.5.5.2 A statement summarizing the strength of the evidence should follow. See Table 4 for a summary of reporting language for Section C pedigrees.

Combined KI of	Reported as
100 or greater	Cannot be excluded
0 to 99	Insufficient support to conclude relatedness

Table 4 - Reporting Language for Section C Pedigrees

5.1.5.5.3 If the reported combined kinship index is greater than one, an additional clarifying statement will be added generally as follows:

"The profile obtained from item 1 is at least 560 times more likely if item 1 is from the cousin of Redacted than if item 1 is from someone unrelated to this individual."

"The profile obtained from item 1 is at least 560 times more likely if item 1 is from the grandparent of Redacted and Redacted than if item 1 is from someone unrelated to these individuals."

¹⁸ A second degree relative is a direct descendant, predecessor, or full sibling of a first degree relative, i.e., a grandparent, grandchild, or full sibling of the parent of the person in question.

¹⁹ A third degree relative is a direct descendant, predecessor, or full sibling of a second degree relative, e.g., a cousin, great-uncle, of the person in question.

5.1.5.5.4 Report Wording Examples for Section C Pedigrees

The qualitative equivalent of the combined KI is based on the magnitude of the reported LR. A request for additional relative samples from the contributor to further refine the relationships is also included. These conclusions should be reported generally as follows:

“Therefore, item 1 cannot be excluded as having originated from Redacted ; however, further testing from at least one additional relative (preferably a parent, child, or full sibling) is necessary to support this conclusion.”*

“These results provide insufficient support to conclude that item 1 originated from Redacted ; however, further testing from at least one additional relative (preferably a parent, child, or full sibling) is necessary.”*

with the following associated explanatory endnote:

*“*These combined kinship index (KI) ranges provide the following support for the conclusion:*

<i>KI</i>	<i>Qualitative Equivalent</i>
<i>≥ 100</i>	<i>cannot be excluded as related</i>
<i>0 to 99</i>	<i>insufficient support to conclude relatedness</i>

However, because of the relationships of the individuals whose samples were provided, this comparison has limited value in drawing conclusions with respect to relatedness.”

5.1.6 Haplotype Results

Autosomal kinship associations can be supported by haplotype results (i.e., Y-STR and/or mitochondrial DNA results).

5.1.6.1 With the approval of the TL, autosomal and haplotype statistics (e.g., Y-STR and/or mitochondrial DNA results) may be combined.²⁰

5.1.6.2 If inclusionary mitochondrial DNA results have been reported, a statement that additional results are available may be included:

“It is noted that the results of the mitochondrial DNA examinations were the subject of a separate report. Please refer to the FBI Laboratory report Redacted dated January 1, 2000 for the results of the mitochondrial DNA examinations.”

Additionally, in the Remarks section of the report, the contributor can be directed to phone the Mitochondrial DNA Examiner to obtain further information.

²⁰ Ge et. al. 2010.

6 Limitations

6.1 It is not possible to anticipate the nature of all potential biological relationships.

6.2 The strength of the combined kinship index is dependent on the References submitted by the contributor. Based on the samples provided and the results obtained, some pedigrees have limited value in determining relatedness.

- When limited genotypes are obtained, a KI may be obtained that has limited value to conclude relatedness even when true relatives are analyzed.
- Section B and C pedigrees have the potential to result in a KI that supports unrelatedness even when true relatives are analyzed. Additionally, there is a potential for fortuitous associations with Section B and C pedigrees.

6.3 KIn CALc software is not validated and will not be used to calculate the KI for relationships beyond first cousins.

6.4 The KI cannot be used to predict the population from which the source of an evidence sample originated.

7 Calculations

See Appendix A.

8 Standards and Controls

Not applicable.

9 Sampling

Not applicable.

10 Measurement Uncertainty

Not applicable.

11 Safety

Not applicable.

12 References

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Rev. #	Issue Date	History
2	11/05/13	<p>Changed “LR” to “KI” for consistency throughout.</p> <p>Changed “smallest” to “lowest” throughout.</p> <p>Updated all figures to account for the new software version.</p> <p>2: Added clarifying wording and added reference to Appendix A.</p> <p>3: Updated KIn CALc Software Version.</p> <p>7.4: Added statement regarding exclusions.</p> <p>7.4.1.1: Added “generally” as not all pedigrees fit this model.</p> <p>7.4.1.2.1.1: Reworded for clarity.</p> <p>Footnote 1: Added amelogenin results are not included in KI.</p> <p>7.4.1.2.2: Added the option to use the Pedigree or the Custom Pedigree tab. The original sections are renumbered for the Pedigree Tab (7.4.1.2.2.1).</p> <p>7.4.1.2.2.1: Added “for Standard Pedigrees”.</p> <p>7.4.1.2.2.1.5, 7.4.1.2.2.1.5.1: New section Pedigree Tab for Non-Standard Pedigrees, including Figure 6.</p> <p>7.4.1.2.2.2: New section Custom Pedigree Tab for Non-Standard Pedigrees, including Figures 7 and 8. Renumbered remaining figures.</p> <p>7.4.1.2.3: Removed references to specific databases.</p> <p>7.4.1.2.3.1, 7.4.1.2.4.1, 7.4.1.2.4.2: Reworded to allow for the Custom Pedigree.</p> <p>7.4.1.2.3.2: Changed “initial screen” to “Profile ID tab”.</p> <p>7.4.1.2.3.3: Revised for clarity. Revised footnote 3 for clarity. Added footnote 4.</p> <p>7.4.1.2.4: Added information regarding which report tab to use.</p> <p>Removed “Report Tab” figure. Reorganized steps within this section for clarity.</p> <p>7.4.1.2.4.4: Reworded for clarity and removed last sentence.</p> <p>7.5: Removed first paragraph as it is repeated from the background section 2. Last paragraph is moved from section 7.6.6 and revised for clarity. Added footnote 9.</p> <p>7.6.2: Revised report wording for clarity.</p> <p>7.6.3: Removed “autosomal” describing Y-STRs.</p> <p>7.6.4: Revised for clarity.</p> <p>7.6.4.1, 7.6.4.2: Moved footnotes into the body of the document.</p> <p>Revised the report wording for clarity.</p> <p>7.6.5.1: Revised the report wording for clarity.</p> <p>7.6.6: Moved first paragraph to 7.5, deleted second paragraph, and renumbered remaining sections.</p> <p>7.6.6.1: Revised footnote 14 for clarity. Removed footnote referencing NRC 1996. Added footnote 15.</p> <p>7.6.6.2: Added option to calculate other PIs. Divided into 7.6.6.2.1, 7.6.6.2.2, and 7.6.6.2.3.</p> <p>7.6.6.2.2: Revised for clarity.</p> <p>7.6.6.2.3: Added last sentence and footnote. Added footnote 16.</p> <p>Revised report and endnote wording for clarity.</p>

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- 7.6.6.3.1: Changed wording in paternity example to more accurately represent the KI.
 - 7.6.6.3.2.1: Revised report wording.
 - 7.6.6.3.2.2: Revised paternity wording for clarity. Changed 99,000 to 99,999 in the endnote wording.
 - 7.6.6.3.2.3, 7.6.6.3.2.4, 7.6.6.3.2.5: Revised endnote wording for clarity. Revised paternity wording for clarity.
 - 7.6.6.4: Changed “will” to “should” for requesting additional samples.
 - 7.6.6.4.2.1, 7.6.6.4.2.3, 7.6.6.4.2.4: Revised endnote wording for clarity.
 - 7.6.6.5: Revised for clarity; changed “will” to “should” for requesting additional samples.
 - 7.6.6.5.3: Changed 100 to 1 for adding an additional clarifying statement.
 - 7.6.6.5.4: Revised body and endnote wording for clarity.
 - 7.6.6.5.5: Changed one to zero and revised endnote wording for clarity.
 - 7.6.7: Added that KIs can be supported by mtDNA and/or Y-STR.
 - 7.6.7.1: Added the option to combine autosomal KIs with haplotype results with TL approval.
 - 7.6.7.2: Revised for clarity.
 - 8: Moved section to Appendix A and renumbered remaining sections.
 - 8.2: Expanded description of limitations.
 - 8.3: Revised for clarity.
 - 8.4: Removed.
 - Appendix A: Moved from previous section 8 with the following modifications:
 - 1.1: Added Table 8 to the listing.
 - 1.5: Added “Combined” to KI; removed the example.
 - 1.6: Removed the example.
- 3 07/27/17 Updated entire document to reflect updated software version and the ability to enter GF loci, including figures.
Removed nDNAU throughout.
Moved N/A sections to the end and renumbered.
- 4.1.2.1: changed theta to 0.01 for most populations and 0.03 for Native American populations.
 - 4.1.2.2: added Linkage section and guidance.
 - 4.1.2.3: added guidance for AT settings for kinship calculations.
 - 4.1.2.4.2: limited the use of the custom pedigree to two scenarios; alternate scenarios require TL approval.
 - 5: removed columns from Table 1, removed Table 2 entirely.
 - 5.1: updated reporting procedures to make similar to current reporting practices for GF interpretation of direct comparisons, including replacing “specimen” with “item”, reporting only the lowest KI/PI, including the verbal scale table in the report .
 - 5.1.3.2: require 4 loci to determine an exclusion.
 - 5.1.4: provide additional guidance for inconclusive profiles, including when a profile is unsuitable for comparisons.

6: Relocated statement regarding what analysis will be conducted from the Limitations to the Background.

6.3, 6.4: added Limitations.

12: Added multiple references.

Appendix A, 1.1: removed tables for hand calculations and cited the appropriate source.

Appendix A, 1.3: update to clarify.

Appendix A, 3.1.3: updated Table to include GF loci and cited appropriate sources.

Approval

Redacted - Signatures on File

Appendix A: Calculations

1 Calculation of Kinship Indices for Single Relative and Parent Samples

1.1 The formulae for the calculation of likelihood ratios that incorporate a coancestry coefficient on a single locus basis for situations involving parents and/or offspring; paternity; reverse paternity; full-siblings; uncle-nephew, half-siblings and grandparent-grandchild; and cousins are found in Ayres, 2000.²¹

1.2 Single-locus KIs are multiplied to obtain the multi-locus kinship index that represents the likelihood of biological relatedness, as follows:

$$\text{Combined KI} = \text{KI}_{\text{LOCUS1}} \times \text{KI}_{\text{LOCUS2}} \times \dots \times \text{KI}_{\text{LOCUS(n)}}$$

1.3 The probability of paternity is calculated using the following formula:

$$= \frac{(\text{CPI} \times \text{Pr})}{[\text{CPI} \times \text{Pr} + (1 - \text{Pr})]}$$

Where CPI = combined PI and Pr = prior probability

The Pr is set to 0.5, which simplifies the probability of paternity to:

$$= \frac{\text{CPI}}{(\text{CPI} + 1)}$$

KIn CALc calculates the probability of paternity and reports the percentage truncated to six significant digits. The examiner will report the probability of paternity, expressed as a percentage, rounded to five significant digits. However, any value greater than 99.999% will be reported as greater than 99.999% and will not be rounded to 100%.

2 Calculation of Kinship Indices when Multiple Relative Samples are Available

2.1 Elston-Stewart

For cases in which multiple alleged relatives (other than parents) are available, the number of iterations of possible genotype combinations becomes too large to be able to calculate by hand. In these situations the software is designed to use the Elston-Stewart algorithm.²²

$$L = \sum_{G_{\text{founder}}} P(X_{\text{founder}} | G_{\text{founder}}) H(G_{\text{founder}}) P(G_{\text{founder}})$$

The Elston-Stewart algorithm considers the probability of founder²³ genotypes and the probability of offspring given the parents.

²¹ Ayres KL. Relatedness testing in subdivided populations, *Forensic Science International* (2000) 114:107-115.

²² This algorithm is described in Human Heredity 21:523-542 (1971).

²³ A founder is a person in the pedigree for which no antecedent genetic information is available.

3 Calculations Involving Mutations

3.1 ‘Ayers’ Mutations Approach

3.1.1 The probability of a maternal allele not mutating is $1 - \mu_{\text{maternal}}$; the probability of a paternal allele not mutating is $1 - \mu_{\text{paternal}}$.

3.1.2 The probability of a maternal allele mutating is $\frac{1}{2} * \mu_{\text{maternal}} * (1/10)^{s-1}$. The probability of a paternal allele mutating is $\frac{1}{2} * \mu_{\text{paternal}} * (1/10)^{s-1}$. In both cases, $\frac{1}{2}$ is the probability of an allele mutating, μ is the mutation rate for the locus in question and s is the number of steps the allele underwent before becoming the “mutated” allele (e.g., if an 11 mutated to 13, $s = 2$).

3.1.3 The mutation rates (μ) for the different loci are found in Table 5 as reported in the AABB 2008 report and in Lu et.al. Int J Legal Med (2012).

	Maternal μ	Paternal μ
CSF1PO	0.000283	0.002021
D10S1248	0	0.0025
D12S391	0.00032	0.003
D13S317	0.000436	0.001743
D16S539	0.000481	0.001127
D18S51	0.000748	0.00253
D19S433	0.000596	0.000745
D1S1656	0	0.0025
D21S11	0.001295	0.001709
D22S1045	0	0.0025
D2S1338	0.000245	0.001526
D2S441	0	0.0025
D3S1358	0.000211	0.001691
D5S818	0.0003	0.001742
D7S820	0.000073	0.001348
D8S1179	0.000333	0.002031
FGA	0.000522	0.003713
SE33	0.00303	0.00639
TH01	0.000043	0.00007
TPOX	0.000081	0.00013
vWA	0.000494	0.003258

Table 5 – Mutation Rates for Various Loci

Procedures for Interpretation of Legacy DNA Data

1 Scope

These procedures apply to DNA personnel who may reinterpret data from a legacy amplification kit and/or report comparisons to previously reported DNA typing results generated using the AmpF/STR® Profiler Plus™, Profiler Plus™ *ID*, COfiler™, Identifiler® Plus and/or MiniFiler™ PCR Amplification Kits that are no longer in use in the DNA Units. In addition, guidance for statistical calculations is included for data generated using legacy amplification kits for which the STRMix Software for statistical analysis is not validated for use.

2 Equipment/Materials/Reagents

PopStats (version 7.0 or higher)

3 Procedures

When comparisons to previously reported DNA typing results are conducted, it may be necessary to reevaluate the DNA data generated using an amplification kit or instrument no longer in use in the DNA units. The data in these instances is referred to as legacy data. If necessary when making conclusions, the reevaluation of any of the allele calls or genotype calls, removal of alleles (or entire loci) from statistical estimates, or a change in the assumptions is considered reinterpretation. DNA examiner must be previously qualified in the interpretation of data from the legacy amplification kit and platform instrument model to perform reinterpretation of legacy data. If necessary, the examiner performing reinterpretation should review the validations that support the legacy results. If reinterpretation is not needed to perform a comparison, the comparison to the previously interpreted data may be performed by any examiner qualified in the technology.

3.1 Application of Peak Height Thresholds to Allelic Peaks

3.1.1 The Peak Amplitude Threshold (PAT) is 50 relative fluorescence units (RFU) for the Profiler Plus™, Profiler Plus™ *ID*, COfiler™, Identifiler® Plus and MiniFiler™ Amplification Kits.

3.1.2 The Match Interpretation Threshold (MIT) is:

- 200 RFU for the Profiler Plus™, Profiler Plus™ *ID* and COfiler™ Amplification Kits
- 200 RFU for the Identifiler® Plus (27 cycles) Amplification Kit
- 300 RFU for the Identifiler® Plus (28 cycles) Amplification Kit
- 300 RFU for the MiniFiler™ Amplification Kit

3.1.2.1 With the exception of the samples types in 3.1.2.2, all the peaks at a given locus must be \geq MIT to be used for matching/statistical purposes. Peaks $<$ MIT may only be used for

purposes of exclusion and/or to establish the presence of a mixture of DNA.

3.1.2.2 For samples known or expected to be of single source origin (e.g., reference samples, alternate reference samples, bones) that display results consistent with having arisen from a single individual, the MIT is applied to only those loci at which stochastic loss of information is possible (i.e., loci that display a single allelic peak < MIT).

3.2 Peak Height Ratios

Peak height ratios (PHR) can be used to associate two alleles to a common source.

3.2.1 Peak height ratios are calculated by dividing the peak height of the allele with the lower RFU value by the peak height of the allele with the higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage.

3.2.2 Tables 1 through 4 describe the minimum expected PHR percentages for the applicable amplification kit. The PHRs for Profiler Plus™ *ID* and COfiler™ are dependent on the detection instrument (i.e., ABI Prism 310 or 3130xl) used. The PHR guidelines are only applicable to allelic peaks that meet or exceed the MIT.

Peak Height	All Profiler Plus™ <i>ID</i> and COfiler™ Loci
200-499 RFU	50%
500-999 RFU	60%
1000 RFU and above	70%

Table 1 - Minimum Expected Heterozygous Peak Height Ratio Guidelines for STR Loci Analyzed Using the Profiler Plus™ *ID* and COfiler™ Kits (3130xl Data)

AmpF/STR Profiler Plus™ and Profiler Plus™ <i>ID</i> Amplification Kit									
Peak Height	D3S1358	vWA	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820
200-300 RFU	60%	60%	55%	60%	60%	55%	60%	60%	60%
301-1000 RFU	65%	65%	65%	65%	60%	60%	65%	65%	65%
Above 1000 RFU	70%	65%	65%	70%	65%	65%	70%	70%	70%

AmpF/STR COfiler™ Amplification Kit						
Peak Height	D3S1358	D16S539	TH01	TPOX	CSF1PO	D7S820
200-300 RFU	60%	60%	60%	60%	60%	60%
301-1000 RFU	65%	65%	65%	60%	60%	65%
Above 1000 RFU	70%	75%	75%	75%	75%	70%

Table 2 - Minimum Expected Heterozygous Peak Height Ratio Guidelines for STR Loci Analyzed Using the Profiler Plus™, Profiler Plus™ ID and COfiler™ Kits (310 Data)

Peak Height	All Identifiler® Plus STR Loci (27 cycles)
200-499 RFU	50%
500-999 RFU	60%
1000 RFU and above	70%

Table 3 - Minimum Expected Heterozygous Peak Height Ratio Guidelines for STR Loci Analyzed Using the Identifiler® Plus (27 cycles) Amplification Kit (3130xl Data)

Peak Height	All MiniFiler™ STR Loci
300-999 RFU	50%
1000 RFU and above	60%

Table 4 - Minimum Expected Heterozygous Peak Height Ratio Guidelines for STR Loci Analyzed Using the MiniFiler™ Amplification Kit

3.2.3 Because reference samples and human remains (e.g., bones, teeth, tissue samples) are attributable to a single individual, PHR assessments are generally not used in their interpretation.

- a. If necessary, however, major contributor types of these samples amplified using Identifiler® Plus (27 cycles) or MiniFiler™ can be assessed using the peak height ratios described.
- b. Major contributor types of these samples amplified using Identifiler® Plus (28 cycles) can be assessed generally using a PHR of 50%.

3.3 Stutter Percentages

The kit-specific stutter percentage guidelines provided in Table 5 are estimates (Average + 3 standard deviations [SD]) of the maximum expected relative stutter values for each locus in the specified amplification kits. These values are expressed as a percentage relative to the source allelic peak height (i.e., % stutter). Although usually observed as N-4, stutter may occur at other locations (e.g., N-8, N+4).

AmpF/STR® Profiler Plus™ ID Amplification Kit

Locus	D3S1358	vWA	FGA	D8S1179	D21S11	D18S51	D5S818	D13S317	D7S820
% Stutter	11	12	12	13	11	14	10	11	13

AmpF/STR® COfiler™ Amplification Kit

Locus	D3S1358	D16S539	TH01	TPOX	CSF1PO	D7S820
% Stutter	11	10	16	13	8	9

AmpF/STR® Identifiler® Plus (27 or 28 cycles) Amplification Kit

Locus	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539
% Stutter	11	12	10	11	13	6	12	11

Locus	D2S1338	D19S433	vWA	TPOX	D18S51	D5S818	FGA
% Stutter	13	13	13	7	15	11	13

AmpF/STR® MiniFiler™ Amplification Kit

Locus	D13S317	D7S820	D2S1338	D21S11	D16S539	D18S51	CSF1PO	FGA
% Stutter	11	11	13	13	13	15	11	12

Table 5 - Maximum Expected Stutter Percentage Guidelines for STR Loci Analyzed Using the Identifiler® Plus and MiniFiler™ Kits

3.4 Interpretation of DNA Typing Results for Single Source Specimens

A sample is generally considered to have originated from a single individual if one or two alleles are present at all loci for which typing results were obtained and the PHR for all heterozygous loci are within the empirically determined values.

3.4.1 For single source specimens, when two peaks at a heterozygous locus each fall into a different peak height category, the PHR will be evaluated relative to the category that corresponds to the peak height of the shorter peak.

3.4.2 A sample that displays a heterozygous peak height imbalance at one or two loci, but for which no other results indicate the presence of a mixture, is generally considered a single-source specimen.¹

¹ Peak height imbalances may be seen in the results from a single individual due to elevated stutter, primer binding site variants that result in attenuated amplification of one allele of a heterozygous pair, or tri-allele patterns in which two copies of an allele are present within the genotype (e.g., a type 11,12,12).

3.4.3 Samples in which three allelic peaks² are observed at a locus, without any other indications of a mixture, may be concluded to be single-source. This conclusion should be based on the relative peak heights of the three peaks and the size range of the alleles that occur at that locus (i.e., larger loci are less likely to display minor contributor alleles for lesser amounts of DNA template).

3.4.4 Single-source samples, including single source fractions from differentially extracted samples, may display a stutter peak(s) that exceeds the guidelines. A peak in a stutter position that exceeds the expected stutter percentage may be interpreted as a stutter peak.

3.5 Interpretation of DNA Typing Results for Mixed Specimens

3.5.1 General Mixture Interpretation Guidelines

Mixtures are generally declared if three or more alleles are present at one or more loci and/or the heterozygous peak height ratios for three or more loci are below expectations. Mixtures can be categorized as distinguishable or indistinguishable. Generally, a multi-locus, mixed sample that contains one or more true minor contributors can be expected to display at least one allelic peak from the minor contributor(s) in a non-stutter position relative to the major contributor.

3.5.1.1 Because locus-specific parameters (e.g., stutter expectations, heterozygous peak height ratios, mutation rate, tri-allelic profile frequencies) may not permit conclusive allelic assignments at a given locus, the classification of any profile as a mixture must be based on an evaluation of the profile in its entirety.

3.5.1.2 The minimum number of contributors to a sample should be estimated, generally by selecting a locus that exhibits the greatest number of allelic peaks. Relative peak heights and possible allelic peak(s) in the stutter position may also be considered when determining the minimum number of contributors.

3.5.1.3 For any pair-wise comparison of peak heights, if two peaks fall into different peak height categories, the PHR will be evaluated relative to the category that corresponds to the peak height of the higher intensity peak.

3.5.1.4 For a mixed sample, including mixed fractions from differentially extracted samples, any peak(s) in a stutter position that exceeds its corresponding expected stutter percentage cannot be interpreted as a stutter peak with certainty and must be concluded to be a possible allelic peak(s).

3.5.1.5 A sample for which two or more stutter peaks exceed their corresponding expected stutter percentages is generally considered a mixture.

² Observed tri-allele patterns are recorded at http://cstl.nist.gov/biotech/strbase/tri_tab.htm.

3.5.2 Deduced Profiles Determined by Subtraction of Expected DNA Typing Results

3.5.2.1 Typing results from conditional reference specimens may be subtracted from the other mixture results to facilitate identification of the foreign alleles. For example, in an apparent two person mixture, a locus has alleles “12, 14, 15, 19” and alleles “12, 14” are attributable to the conditional reference specimen, the extrinsic alleles “15, 19” are attributable to a different individual.

3.5.2.2 If sharing of alleles among the donor of the conditional reference specimen and an additional individual is suspected, any separation of each individual’s alleles must be based on quantitative differences in allelic peak heights at a given locus. For example, given alleles “12, 13, 14” with respective peak heights of 800, 1000, and 200 RFU, although the peak height ratio of alleles “12” and “13” is within expectations (80%), it is possible that the “13” allele is shared by both individuals. If the DNA typing results from the conditional reference specimen are “12, 13” then the possible genotypes for the unknown contributor to this mixture result are either “13, 14” or “14, 14.” Using this assignment strategy, the genotype for the unknown contributor cannot be further refined.

3.5.2.3 The extrinsic alleles may effectively constitute a single-source profile (i.e., there is one DNA contributor in addition to the individual from whom the specimen was taken) or a mixture profile (i.e., there are multiple DNA contributors in addition to the individual from whom the specimen was taken). Regardless, the remaining alleles should be assessed using mixture guidelines for PHR assessments (i.e., use the taller peak to determine the PHR category) and stutter (i.e., consider elevated stutter peaks as possibly allelic).

3.5.2.4 This approach can be used when another known individual can be expected to have contributed biological material to the mixed specimen (i.e., consensual partner). In such situations, the strategies given for the subtraction of the DNA typing results of a single conditional reference specimen from any extrinsic alleles present should be applied for both of the conditional reference specimens (i.e., victim and consensual partner).

3.5.2.5 This approach can be applied to evidentiary items from which DNA is isolated by means of a differential extraction. The strategies given for the separation of the typing results of a conditional reference specimen(s) from any extrinsic alleles present in an appropriate mixed question specimen may be applied to a mixed result obtained from either the female and/or male fractions. In such situations, the single-source or major contributor typing results from one fraction may be used as a conditional reference specimen(s) to the complementary fraction.

3.5.3 Deduced Single-Source Profiles Determined from Distinguishable Mixtures

3.5.3.1 Interpretation of the results for the major and/or minor contributors may be limited to only some loci.

3.5.3.2 The major contributor can be determined to be heterozygous at a locus if the two alleles of greatest amplitude at a given locus meet PHR expectations and if no pair-wise comparison(s) of the other allelic peak heights at the locus meet PHR expectations.

3.5.3.3 The major contributor can be determined to be homozygous at a locus if the allelic peak that displays the greatest height is not in PHR with any other allelic peak when this peak is considered as both a single allelic dose (i.e., total peak height) and considered as two hypothetical allelic doses with heights at the extremes of the appropriate PHR expectation range.

3.5.3.3.1 The purpose of this assessment is to consider the tallest peak observed at a locus in terms of two separate amplifications of individual gametic contributions of the same allele. By considering the observed peak in this way, the potential homozygous genotype is effectively considered as simply a special type of heterozygote (i.e., maternal and paternal contributions of a like allele) whose allelic pair displays the minimum allowable PHR. Each dose of this hypothetical pair is then compared to the other allelic peak heights at the locus to determine if either or both are in PHR expectation with any other observed allele.

3.5.3.3.2 To determine the peak heights of the two hypothetical homozygous doses of the tallest allele detected at the locus, start with the equation:

$$H_{\text{Peak A}} = H_{\text{Observed}} / (1 + \text{PHR}_{\text{Minimum}})$$

where $H_{\text{Peak A}}$ is the calculated height of the first hypothetical dose, H_{Observed} is the observed peak height of the tallest allele detected at the locus, and $\text{PHR}_{\text{Minimum}}$ is the minimum expected PHR value for the half-height of the tallest allele prescribed by Tables 6 and 7. Then use the equation:

$$H_{\text{Peak B}} = H_{\text{Observed}} - H_{\text{Peak A}}$$

where $H_{\text{Peak B}}$ is the calculated height of the second hypothetical dose.

For example, given a potential distinguishable mixture result at a locus with the alleles “12, 13, 14, 15, 16” with respective peak heights of 78, 800, 2500, 149, and 200 RFU, while the peak height ratio of allele “14” is not within PHR expectation with any other allelic peak based on its H_{Observed} , this peak must also be evaluated as a pair of individual hypothetical homozygous doses. Using the equations above, allelic peak “14” has a half-height of 1250 RFU which requires the use of a minimum expected heterozygous peak height ratio of 70% (i.e., 0.70) which yields one dose of peak height:

$$H_{\text{Peak A}} = H_{\text{Observed}} / (1 + \text{PHR}_{\text{Minimum}}) = 2500 \text{ RFU} / (1 + 0.70) = 1470 \text{ RFU}$$

and a second dose of height:

$$H_{\text{Peak B}} = H_{\text{Observed}} - H_{\text{Peak A}} = 2500 \text{ RFU} - 1470 \text{ RFU} = 1030 \text{ RFU}$$

Given this hypothetical homozygous dose pair, because Peak B is in PHR expectation (77%) with the observed “13” allele (the second tallest observed allelic peak), the genotype “14, 14” is not the only possible genotype for the major contributor.

3.5.3.4 The DNA typing results attributed to the minor contributor must also meet PHR expectations in order to be used in a random match probability calculation.

3.6 Application of Statistics

3.6.1 The random match probability (RMP)^{3,4} may be used to calculate the multi-locus genotype frequency for single-source⁵ or deduced single-source⁶ (i.e., major contributor, minor contributor, deduced extrinsic contributor) matches. The combined probability of inclusion (CPI) may be used to calculate the multi-locus genotype frequency for a mixture. The CPI is used for indistinguishable mixtures. The CPI may be used to represent the minor contributor(s) in a distinguishable mixtures, where the minor contributor(s) cannot be refined to a single genotype.

3.6.2 No statistics are calculated for exclusionary or inconclusive conclusions.

3.6.3 Statistics must be calculated when a positive association is made between an evidentiary sample and a reference sample.

3.6.3.1 A match between an intimate sample and the associated reference sample does not require a statistic. The provenance of the intimate sample must be established in the case record when statistics are not calculated.

3.6.3.2 For a given sample, more than one set of statistics may be calculated and reported. When appropriate, statistics should be calculated and reported with and without relevant assumptions (e.g., a victim's DNA types can be reasonably expected to be on her bed sheet). Any assumptions used in an interpretation must be clearly stated in the report.

3.6.4 Composite DNA profiles for samples may be used for matching/statistical purposes. To reasonably ensure that a profile compiled from genetic information derived from separate extractions, amplifications, and/or injections has arisen from the same individual, the resultant profile must 1) be compiled from different items from a common source (e.g., replicate vaginal swabs), multiple cuttings of the same evidentiary stain, or cuttings from different stains of the same grouping on a given evidence item and 2) demonstrate concordance at the available redundant loci.

3.6.5 RMPs and CPIs⁷ are calculated using four general United States population groups (African American, Caucasian, Southeastern Hispanic, and Southwestern Hispanic). Additional RMPs or CPIs will be reported for matching specimens that potentially originate from Native

³ The sex typing results from the amelogenin locus are not included in the random match probability calculations.

⁴ Any triallelic locus is not included in the statistical calculations, but may be used for exclusionary purposes.

⁵ For samples determined to be single-source, loci that do not meet PHR expectations may be used for matching/statistical purposes.

⁶ For deduced single-source samples, loci that do not meet PHR expectations may not be used in the RMP calculation.

⁷ All random match probabilities and combined probabilities of inclusion will be reported as fractions truncated to two significant figures.

American populations (i.e., Apache, Minnesota Native American, and Navajo), Caribbean populations (i.e., Trinidadian), or Chamorro/Filipino populations.⁸

3.6.5.1 The allele frequency distributions for the African American, Caucasian, Southeastern Hispanic, Southwestern Hispanic, Apache, Navajo, Trinidadian, Chamorro, and Filipino populations are published in *Forensic Science International: Genetics*.⁹ The allele frequency distributions for the Minnesota Native American population are included in the applicable DNA procedure for interpretation of GlobalFiler™ data (i.e., DNA 233). The African American population includes samples from the African American, Bahamian, and Jamaican populations.

3.6.5.2 Examiners may use other published allele frequency distributions for the calculation of RMPs or CPIs in cases potentially involving other defined, dissimilar population groups.

3.7 Suggested Reporting Language

The results and/or conclusions for specimens subjected to DNA analysis will generally be reported in narrative form. The formatting and administrative information required in a report are described in the appropriate *FBI Laboratory Operations Manual* practices and the *DNA Procedures Manual*. For additional guidance on reporting language refer to the appropriate interpretation protocols of the *DNA Procedures Manual*.

3.7.1 Comparisons to Previously Reported Results

A statement should be included to identify item(s) previously subjected to DNA typing but for which the results of additional comparisons should be reported as follows:

“The STR typing results from items 1 and 2 were compared to the STR typing results from item 3 (SMITH) [submitted under FBI Laboratory Number 2014-01234 and reported in FBI Laboratory report dated July 26, 2014].”

3.7.2 Exclusion (No-Match) Conclusions

3.7.2.1 Exclusions from Single Source Specimens

An exclusion is declared when upon comparison of a reference specimen to a single-source specimen, the profiles are found to be different at one or more loci.¹⁰ An exclusionary conclusion should be reported as follows:

⁸ The use of Native American, Caribbean, or Chamorro/Filipino population databases is generally based on the geographic location of the requesting agency. The listed Native American or Caribbean population databases are appropriate for use regardless of the specific Native American or Caribbean population group in the case scenario. Statistics for cases originating from Puerto Rico will be calculated using the four general United States population databases and do not require the use of the Caribbean population databases.

⁹ Moretti TR, Moreno LI, Smerick JB, Pignone ML, Hizon R, Buckleton JS, Bright J-A, Onorato AJ. Population data on the expanded CODIS core STR loci for eleven populations of significance for forensic DNA analyses in the United States. *Forensic Science International: Genetics* (2016) 25: 175-181.

¹⁰ Stochastic loss of allelic peaks may occur at loci that exhibit peak heights below the MIT. Therefore, the absence of an allele at such a locus may not be exclusionary.

“Item 2 (SMITH) is excluded as a potential contributor to item 1.”

3.7.2.2 Exclusions from Mixed Specimens

A mixture exclusion is declared when upon comparison of a reference specimen to a mixed specimen either (a) one or more alleles between the mixed specimen and reference specimen are different¹¹ or (b) for any given locus, the reference specimen genotype is not consistent with any potential allele combinations in the mixture.¹² An exclusionary conclusion should be reported as follows:

“DNA from two or more individuals was obtained from item 1. Item 2 (SMITH) is excluded as a potential contributor to item 1.”

3.7.3 Inclusion (Match)¹³ Conclusions

3.7.3.1 Matches to Single Source Specimens

A match is declared when upon comparison of a reference specimen to a single-source specimen, the profiles are found to be concordant at all loci for which interpretable results were obtained. Single-source match conclusions that fit the following scenarios should be reported as outlined:

RMP that does not meet source attribution:

“The DNA profile from item 1 matches^A item 2 (SMITH). The random match probabilities^B calculated for item 1 are approximately:

*1 in 1 million from the African American population
1 in 11 million from the Caucasian population
1 in 100,000 from the Southeastern Hispanic population
1 in 120,000 from the Southwestern Hispanic population.”*

Source attribution:

“Item 2 (SMITH) is the source^C of the DNA obtained^B from item 1.”

Matches to a conditional reference samples (For intimate samples):

¹¹ Stochastic loss of allelic peaks may occur at loci that exhibit peak heights below the MIT. Therefore, the absence of an allele at such a locus may not be exclusionary.

¹² If a peak is not considered allelic because it is below the stutter threshold, the absence of that allele at such a locus may not be exclusionary.

¹³ As used here, “match,” “inclusion,” “included,” and “cannot be excluded,” (as well as any other variations on such language), are used synonymously and are not intended to capture differing degrees of similarity between two DNA profiles or to imply increased/decreased meaning to a particular set of DNA profiles that are found to be indistinguishable.

“No STR typing results unlike item 2 (SMITH) were obtained from item 1.”

“No STR typing results unlike item 2 (SMITH) were obtained from item 1; therefore, no comparisons were made to item 3 (JONES).”

The associated endnotes represented by the superscript letters are listed in section 3.7.3.3.

3.7.3.2 Inclusions to Mixed Specimens

A mixture inclusion is declared when upon comparison of the DNA profile from a reference specimen to a mixed specimen, the genotype of the reference specimen is consistent with one of the possible genotypes present in the mixture. It is noted that one or more of the reference specimen's alleles may not be detectable in the mixture [e.g., due to stochastic loss of an allele(s) or a peak that is below the stutter threshold]. Mixed specimens that fit the following scenarios should be reported as outlined:

Mixture conclusions are generally preceded with a statement regarding the number of potential contributors:

“DNA from two or more individuals was obtained from item 1.”

When the results could have originated from a single individual or a mixture, based on the nature of the results, the number of potential contributors may be expressed as:

“DNA from at least one individual was obtained from item 1.”

Major/minor contributor RMP that does not meet source attribution:

“Item 2 (SMITH) is potentially the major contributor of the DNA obtained from item 1. The random match probabilities^B calculated for item 1 are approximately:

*1 in 1 million from the African American population
1 in 11 million from the Caucasian population
1 in 100,000 from the Southeastern Hispanic population
1 in 120,000 from the Southwestern Hispanic population.”*

Major/minor contributor that meets source attribution:

“Item 2 (SMITH) is the source of the major contributor^C of the DNA obtained^B from item 1.”

Combined probability of inclusion:

“Items 2 (SMITH) and 3 (JONES) cannot be excluded^A as potential contributors to this mixture. The probabilities of inclusion^D calculated for item 1 are approximately:

*1 in 120,000 from the African American population
1 in 440,000 from the Caucasian population
1 in 90,000 from the Southeastern Hispanic population
1 in 99,000 from the Southwest Hispanic population.”*

Alternatively, this information can also be reported with the exclusionary only and/or matching loci delineated:

“Based on the STR typing results at the genetic loci D3S1358 and D5S818, item 2 (SMITH) cannot be excluded...”

When the probability of inclusion estimate results in a frequency which is equivalent to approximately “1 in every 1 individuals”, this estimate should be reported as noted above, but also with the percentage (i.e., 67%, rounded to two significant figures) ¹⁴ on which the “1 in 1” estimate is based.

“1 in 1 from the African American population, which includes approximately 72% of this population as potential contributors;

1 in 1 from the Caucasian population, which includes approximately 72% of this population as potential contributors;

1 in 1 from the Southeastern Hispanic population, which includes approximately 81% of this population as potential contributors;

1 in 1 from the Southwestern Hispanic population, which includes approximately 81% of this population as potential contributors.”

Unknown major with a CPI on the minor:

“A major contributor can be discerned from item 1 and is suitable for comparison purposes. Item 2 (SMITH) is excluded as a potential major contributor of the DNA recovered from item 1; however, item 2 (SMITH) cannot be excluded^A as a potential minor contributor to this mixture. The probabilities of inclusion^D calculated for item 1 are approximately: ...”

Mixture results in which reference profiles can be subtracted out, the sample is intimate, and the provenance of the sample is established in the case record:

“The DNA profile unlike item 2 (JONES) obtained from item 1 matches^A item 3 (SMITH). The random match probabilities^B calculated for item 1 are approximately:...”

¹⁴ The composite frequency (e.g., 6.829E-01) is converted into a percentage, which is rounded to two significant figures. For example, in the traditional manner, 67.49% is rounded down to 67%, and 67.51% is rounded up to 68%. However, when the decimal value is exactly 0.50%, rounding is performed according to the “round half up” rule, whereby, for example, 67.50% is rounded up to 68%.

“No STR typing results unlike item 2 (SMITH) were obtained from the major contributor to item 1.”

“The DNA profile unlike item 2 (JONES) obtained from item 1 indicates the presence of at least two individuals. Item 3 (SMITH) is potentially the major contributor of the DNA obtained from item 1. The random match probabilities^B calculated for item 1 are approximately: ...”

Mixture results in which a reference profile(s) is subtracted out and the assumption of an expected contributor is stated in the report:

“Assuming item 2 (JONES) is a contributor,^E the DNA profile unlike item 2 (JONES) obtained from item 1 matches^A item 3 (SMITH). The random match probabilities^B calculated for item 1 are approximately:...”

“Assuming item 2 (JONES) is a contributor,^E the DNA profile unlike item 2 (JONES) obtained from item 1 indicates the presence of at least two individuals. Item 3 (SMITH) is potentially the major contributor of the DNA obtained from item 1. The random match probabilities^B calculated for item 1 are approximately: ...”

A final statement may be included if the reference samples can or cannot account for all the typing results:

“It is noted that items 2 (SMITH) and 3 (JONES) can/cannot account for all of the DNA typing results obtained from item 1.”

3.7.3.3 Associated Endnotes for Inclusionary Reporting Language

“^A As used here, the terms “match” or “cannot be excluded” are used synonymously, are not intended to capture differing degrees of a conclusion and are not used to capture or convey any information regarding how common or rare a particular DNA profile is in a population.”

“^B Not all loci at which amplification is attempted will yield conclusive results; a statistical estimate (random match probability) has been based on loci with conclusive typing results for which allelic frequency data is available. The random match probability is defined as the chance of selecting an unrelated individual at random having an STR profile matching the DNA obtained from the evidence item. The uncertainty associated with a random match probability has been empirically demonstrated to be less than 10-fold in either direction.”

“^C This opinion is based upon the outcome of a statistical calculation in which the probability of selecting an unrelated individual at random from an African American, Caucasian, Southeastern Hispanic, or Southwestern Hispanic population having a DNA profile matching the contributor of the DNA obtained from the evidence item(s)

was determined to be equal to, or less than, 1 in 7 trillion individuals. It is noted that the African American population used for calculations includes samples from the African American, Bahamian, and Jamaican populations.”

If statistics are calculated for Native American populations and source attribution is reported, the following should be added to the endnote above:

“C...and equal to, or less than, 1 in 4.3 billion individuals in the Apache, Minnesota Native American, or Navajo population.”

3.7.4 Inconclusive Results

An inconclusive conclusion is declared when the DNA typing results are declared not suitable for matching/statistical purposes (i.e., suitable for exclusionary purposes only), and the comparison does not result in an exclusion. A single-locus or multi-locus DNA typing result can be declared not suitable for matching/statistical purposes when the peak height of an allele(s) falls below the MIT. It is noted that inconclusive may also refer to individual locations whose peaks cannot be resolved. If peaks cannot be resolved at a locus, that locus should not be used for comparison purposes.

3.7.4.1 Inconclusive Results in a Single-Source Specimen

3.7.4.1.1 Loci are deemed not suitable for matching/statistical purposes based on the MIT of the amplification kit used and the sample type analyzed.

3.7.4.1.2 If all loci are declared not suitable for matching/statistical purposes, then the DNA profile may be used for exclusionary purposes only. Any comparison not resulting in an exclusion should be declared inconclusive and reported as follows:

“The STR typing results for item 1 may only be used for exclusionary purposes.^F Item 2 (SMITH) is excluded as a potential contributor. Item 3 (JONES) is inconclusive^G with regards to the comparison to item 1.”

The STR typing results obtained for item 1 may only be used for exclusionary purposes;^F however, no DNA unlike item 2 (SMITH) was obtained from item 1.”¹⁵

The associated endnotes are listed in the Associated Endnotes for Inconclusive Reporting Language section below.

3.7.4.2 Inconclusive Results in a Mixed Specimen

3.7.4.2.1 In a distinguishable mixture, the allelic peaks for the major and minor contributor(s) are evaluated separately. For a given potential contributor, a locus cannot be used for

¹⁵ This statement is used to capture inconclusive associations between an intimate sample and the associated reference sample.

matching/statistical purposes if any allele is below the MIT. For example, if the mixed specimen exhibits a major contributor type “15, 16” and the minor peaks are “12, (13)” where the “13” allele is <MIT, the alleles of the major contributor are interpretable, but the minor contributor is not suitable for matching/statistical purposes at this locus. If all loci attributable to the major or minor contributor are not suitable for matching/statistical purposes, any comparison not resulting in an exclusion is declared inconclusive with regard to the respective contributor. This scenario may be reported as follows:

“DNA from two or more individuals was obtained from item 1. A major contributor can be discerned and is suitable for matching purposes. The STR typing results for the minor contributor(s) to item 1 may only be used for exclusionary purposes.^F Item 2 (SMITH) is excluded as a potential major contributor; however, item 2 (SMITH) is inconclusive^G with regards to the comparison to the minor contributor to item 1.”

3.7.4.2.2 For an indistinguishable mixture, all allelic peaks are considered collectively for the purpose of determining loci that are not suitable for matching/statistical purposes. If any locus contains an allelic peak below the MIT, the locus is declared not suitable for matching/statistical purposes. For example, at a given locus, if peaks “(12), 13, 14, 15” are detected with peak “12” <MIT, this locus would be deemed not suitable for matching/statistical purposes. If all loci exhibit one or more allelic peaks that are below the MIT, then the entire mixture is not suitable for matching/statistical purposes. Any comparison not resulting in an exclusion is declared inconclusive and reported as follows:

“DNA from two or more individuals was obtained from item 1. These results may only be used for exclusionary purposes.^F Item 2 (SMITH) is inconclusive^G with regards to the comparison to item 1.”

3.7.4.2.3 Associated Endnotes for Inconclusive Reporting Language

“^F When the potential exists that not all of the genetic information in a biological sample has been detected, the results are not suitable for matching purposes; however, they may be used for exclusionary purposes. For STR typing results to be used for matching purposes, sufficient DNA quality and/or quantity is necessary.”

“^GA comparison is inconclusive when the reference sample can be neither included nor excluded as a potential contributor.”

4 Calculations

The calculations described are generally accomplished with the aid of the FBI Popstats software.

4.1 Calculation of Random Match Probabilities

4.1.1 For a heterozygous locus A with alleles A_i and A_j , the single-locus genotype

frequency¹⁶ (P_{ij}) is twice the product of the frequencies of occurrence of the allele A_i (p_i) and allele A_j (p_j) of the heterozygous type.

As an example, for the heterozygous type “22, 24”, in which the frequency of occurrence for the “22” allele is 0.2250 and the “24” allele is 0.1861, the single-locus genotype frequency estimate is as follows:

$$P_{ij} = 2 p_i p_j = (2)(0.2250)(0.1861) = 0.0837$$

4.1.2 For a homozygous locus, the single-locus genotype frequency (P_{ii}) is calculated using the following expression:

$$P_{ii} = p_i^2 + [p_i(1-p_i) \Theta]$$

where p_i represents the frequency of occurrence of allele A_i , and the parameter Θ is an estimate of the effects of population subdivision. A Θ value of 0.01 is routinely used for populations such as African American, Caucasian, Southeastern Hispanic, Southwestern Hispanic, Chamorro, Filipino, and Trinidadian. For populations such as Native Americans (i.e., Apache, Minnesota Native American, and Navajo), a Θ value of 0.03 is employed.

As an example, for the homozygous type “22, 22” in which the frequency of occurrence for the “22” allele is 0.2250, the homozygous expression would yield the following single-locus genotype frequency estimate:

$$\begin{aligned} P_{ii} &= p_i^2 + [p_i(1-p_i) \Theta] \\ &= (0.2250)^2 + [(0.2250)(1-0.2250)(0.01)] = 0.0524 \end{aligned}$$

4.1.3 A single-locus genotype frequency ($P_{\text{LOCUS}(n)}$) obtained for a matching single-source sample can be multiplied by the frequency(ies) found for the same sample at other matching STR loci to obtain a combined multi-locus genotype frequency (P_{PROFILE}) estimate as follows:

$$P_{\text{PROFILE}} = P_{\text{LOCUS}1} \times P_{\text{LOCUS}2} \times \dots \times P_{\text{LOCUS}(n)}$$

4.2 Calculation of the Combined Probability of Inclusion (CPI)

4.2.1 For an indistinguishable mixture, only those loci for which all of the alleles detected are above the MIT are included in this calculation.

4.2.2 The frequencies of occurrence (e.g., $p_1, p_2, p_3, \dots, p_n$) of all the alleles present at the matching locus (A_n) are summed and then this sum is squared, as follows:

¹⁶ The term frequency is used customarily to refer to a relative frequency, or proportion. The lowercase “p” refers to an allelic frequency, and the uppercase “P” refers to a genotypic frequency.

$$P_{\text{LOCUS}} = (p_1 + p_2 + \dots p_n)^2$$

to yield the probability of an unrelated individual having a single-locus genotype that would be observed within the mixture result obtained from that locus (P_{LOCUS}).

4.2.3 The single-locus probabilities (e.g., $P_{\text{LOCUS}1}$, $P_{\text{LOCUS}2}$, ..., $P_{\text{LOCUS}n}$) for all loci (A_n) for which the alleles are at or above the MIT are then multiplied to yield the probability of an unrelated individual having a multi-locus genotype that would be observed within the mixture result obtained from that specimen. This value is the combined probability of inclusion (CPI).

$$\text{CPI} = P_{\text{LOCUS}1} \times P_{\text{LOCUS}2} \dots \times P_{\text{LOCUS}(n)}$$

4.2.4 To calculate the combined probability of exclusion (CPE), the CPI is subtracted from 1. The CPE may be multiplied by 100% to express it as the percentage of the population excluded as a donor to the mixture of DNA obtained from the specimen.

$$\text{CPE} = (1 - \text{CPI}) \times 100\%$$

4.3 Source Attribution

4.3.1 The source of a known specimen can be concluded to be the source of a single-source or major/minor contributor questioned profile (with the exception of monozygotic twins), if the adjusted RMP (p_1) for unrelated individuals of the STR typing results found to be shared by the matching reference and questioned specimen(s) satisfy the following condition:

$$p_1 \leq 1 - (1 - \alpha)^{1/N}$$

where α is the level of significance (confidence level), and N is the estimated size of the population of interest. For forensic casework, a 0.99 confidence level is used ($\alpha = 0.01$), and the population of interest is that of the world ($N = 7$ billion).

4.3.2 The adjusted probability (p_1) is based on the RMPs calculated for the single-source or major/minor contributor STR typing results, and is the RMP increased by a factor of 10.¹⁷ Incorporating this value (10 x RMP) into the above formula yields:

$$10 \times \text{RMP} \leq 1 - (1 - \alpha)^{1/N}$$

Which simplifies to:

$$\text{RMP} \leq \frac{1 - (1 - \alpha)^{1/N}}{10}$$

4.3.3 Therefore, the source attribution threshold for forensic casework is 1.4E-13 (or 1 in 7

¹⁷ This adjustment was recommended by the National Research Council in their 1996 report entitled "The Evaluation of Forensic DNA Evidence," pages 156 and 160.

trillion) for the four general population groups (i.e., African American, Caucasian, Southeastern Hispanic, and Southwestern Hispanic). This threshold is also used for smaller non-Native American populations (e.g., Trinidadian, Chamorro, and Filipino).

For Native American populations, the estimated size of the combined Native American, Eskimo, and Aleut populations ($N = 4.3$ million) is used in lieu of the world's population,¹⁸ which results in a source attribution threshold for Native American populations of $2.3E-10$ (or 1 in 4.3 billion).

4.3.4 If all of the calculated RMPs are less than or equal to the thresholds indicated above, source attribution can be reported. If any of the calculated RMPs are more than the calculated thresholds, RMPs for all calculated populations will be reported.

4.3.5 If source attribution is declared for a sample no additional comparisons to that sample are necessary.

4.4 Minimum Allele Frequencies

4.4.1 A minimum allele frequency (p_{MINIMUM}) estimation is substituted for the calculated frequency for rare alleles at an STR locus. For purposes of using a minimum allele frequency, a rare allele at a given locus is defined as one that is found to occur ≤ 4 times in a population database. The use of a minimum allele frequency establishes a lower limit for the frequency of an allele at a locus and ensures that the frequency of occurrence of a rare allele is not underestimated.

4.4.2 The minimum allele frequency (p_{MINIMUM}) for the rare allele at a locus is established using the following equation:

$$p_{\text{MINIMUM}} = 5/2N$$

where N is the number of individuals making up a particular database (i.e., the number of individuals whose DNA types were included in the database for a given locus).

4.4.3 The minimum allele frequency will be used for microvariants and other rare alleles that are not included in the Allele Frequency Databases.

5 Standards and Controls

Not applicable.

¹⁸ The population estimate of 4.3 million is the projected size of the combined Native American, Eskimo, and Aleut populations in the year 2050 and was taken from the United States Census Bureau.

6 Sampling or Sample Selection

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 These procedures describe the parameters that should be used in conjunction with the appropriate interpretation and reporting procedures of the *DNA Procedures Manual*. Refer to the *DNA Procedures Manual* for additional information regarding the limitations of interpretation.

8.2 It is not possible to anticipate the nature of all potential DNA typing results or the nature of the evidentiary specimens from which they may be obtained. These procedures do not exhaust the possible list of the results that may be encountered by the Examiner nor the conclusions that an Examiner may render based on his/her interpretation of those results. For those results not specifically described, conclusions should be drawn using the procedures given for the results above that are similar in concept and/or origin.

9 Safety

Not applicable.

10 References

FBI Laboratory Quality Assurance Manual

FBI Laboratory Operations Manual

DNA Procedures Manual

2015 FBI Population Data for the expanded CODIS core STR loci. Available at: <https://www.fbi.gov/about-us/lab/biometric-analysis/codis/expanded-fbi-str-2015-final-6-16-15.pdf>.

Applied Biosystems. *AmpFlSTR® Profiler Plus® PCR Amplification Kit User's Manual*, P/N 4303501G, Foster City, CA.

Applied Biosystems. *AmpFlSTR® Profiler Plus® ID PCR Amplification Kit User Bulletin*, P/N 2330429 Rev. D, Foster City, CA.

Applied Biosystems. *AmpFlSTR® COfiler® PCR Amplification Kit User Bulletin*, P/N 4306116
Rev. E, Foster City, CA.

National Research Council. *The Evaluation of Forensic DNA Evidence*, Washington, DC.:
National Academy Press, 1996.

Rev. #	Issue Date	History
0	04/24/13	Original document issued. Information previously contained within the Short Tandem Repeat Analysis Protocol (Archived 7-24-06) and DNA SOP 216-5.
1	02/28/18	Added interpretation, reporting, and statistical calculation guidance previously contained in DNA 229-2 which was superseded on 12/01/15 with the implementation of STRMix. Updated references from nDNAU to DCU or DNA throughout. Added guidance about reinterpretation and legacy data.

Redacted - Signatures on File

Approval

DNA Technical Leader

Date: 02/27/2018

DCU Chief

Date: 02/27/2018

BAU Chief

Date: 02/27/2018

QA Approval

Quality Manager

Date: 02/27/2018

Procedures for the Manual Extraction of DNA

1 Scope

These procedures apply to DNA personnel performing manual extraction of deoxyribonucleic acid (DNA) using Phenol/Chloroform/Isoamyl Alcohol (PCIA) and Microcon[®] filters in the DNA Casework Unit (DCU) or Biometrics Analysis Unit (BAU).

2 Equipment/Materials/Reagents

Materials

- General laboratory supplies (e.g., tubes, pipettes)
- Costar[®] spin baskets, or equivalent (*for differential extractions*)
- Qiagen[®] Lyse & Spin Baskets and Collection Tubes, or equivalent (*for normal extractions*)
- Phase Lock Tubes, 1.5 mL or 2 mL (Phase Lock Gel [™] Low or High Density Gel, Qiagen[®] MaXtract High Density, or equivalent)
- Microcon[®] DNA Fast Flow Centrifugal Filter Device and Tubes (EMD Millipore Corporation or equivalent)

Reagents

- 25:24:1 Phenol/Chloroform/Isoamyl Alcohol (PCIA)
- Proteinase K, 20 mg/ml
- Water, Reagent Grade or equivalent
- Stain Extraction Buffer (SEB) with Dithiothreitol (DTT) (*for normal extractions*)
- Dithiothreitol (DTT), 1M (*for normal extractions*)
- Sarkosyl, 20 mg/ml (*for differential extractions*)
- Sperm Wash Buffer (*for differential extractions*)
- TNE Buffer (TNE) (*for differential extractions*)

Refer to the appropriate DNA procedure (i.e., DNA QA 609) for reagent preparation information.

3 Standards and Controls

At least one extraction control (i.e., reagent blank) must be processed in parallel with each extraction batch.

For evaluation of the extraction controls, refer to the appropriate interpretation procedure of the *DNA Procedures Manual*.

4 Procedures

Refer to the DNA Procedures Introduction (i.e., DNA QA 600) for applicable general precautions and cleaning instructions.

Ensure the appropriate fields (i.e., instruments, reagents) in STACS are completed from any network computer, as necessary.

4.1 Normal Extraction Lysis

4.1.1	Create master mix for the extraction batch.	
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Normal Extraction Master Mix

Reagent	μL per sample
SEB w/DTT	450
Pro K	3

4.1.2	Add 450 μL master mix.	
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4.1.3	Vortex, quick spin and incubate with agitation at 56°C for 2-4 hours.	
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4.1.4	Quick spin. If necessary, transfer cutting to an appropriate basket, spin (generally between 9,000 and 13,000 rpm for 5 minutes), discard basket.	
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Proceed to PCIA and Microcon Purification, Section 4.3.

4.2 Differential Extraction Lysis, Fractionation, and Sperm Wash

4.2.1	Create master mix for the extraction batch.	
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Differential Extraction Master Mix

Reagent	μL per sample
TNE	400
Sarkosyl	25
Reagent grade water	75
Pro K	1

4.2.2	Add 450 μL master mix.	
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4.2.3	Vortex, quick spin and incubate with agitation at 37°C for 2-4 hours.	
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4.2.4	Quick spin. If necessary, transfer cutting to an appropriate basket, spin (generally between 9,000 and 13,000 rpm for 5 minutes), discard basket.	
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4.2.5	Remove the supernatant, avoiding the pellet, and transfer it into a new labeled microcentrifuge tube.	
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The supernatant is the epithelial (F) fraction. The cell pellet remaining in the tube is the sperm (M) fraction.

Processing of the F fraction resumes at PCIA and Microcon Purification, either independently or with the M fraction.

4.2.6	Add 450 μ L Sperm Wash Buffer to the M fraction.	
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4.2.7	Vortex and spin (generally between 9,000 and 13,000 rpm for 5 minutes).	
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4.2.8	Remove and discard the supernatant, avoiding the pellet.	
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4.2.9	Repeat sperm wash steps (4.2.6, 4.2.7, and 4.2.8) two additional times.	
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4.2.10	Ensure the M fraction master mix has been created for the extraction batch.	
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M Fraction Master Mix

Reagent	μ L per sample
TNE	225
Sarkosyl	75
Reagent grade water	225
DTT	10.5
Pro K	3

4.2.11	Add 450 μ L M fraction master mix to M fraction tubes.	
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4.2.12	Vortex, quick spin and incubate with agitation at 37°C for 2-4 hours.	
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Proceed to PCIA and Microcon Purification.

4.3 PCIA and Microcon Purification

The PCIA should be allowed to equilibrate to room temperature prior to use.

If differential extracts are processed simultaneously, the M fractions and their corresponding reagent blanks are processed through each step of the purification prior to the F fractions.

4.3.1	If needed, quick spin all tubes. In a fume hood, add 450 µL PCIA to each tube.	
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Dispose of PCIA and all consumables (i.e., tips, tubes) that come into contact with PCIA in an appropriate waste container.

4.3.2	Spin the phase lock tubes (generally between 9,000 and 13,000 rpm for 30 seconds) to pellet the phase lock gel. Vortex, quick spin and add entire volume of PCIA/lysate emulsion to pelleted, labeled phase lock tube.	
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4.3.3	Spin (generally between 9,000 and 13,000 rpm for 5 minutes).	
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4.3.4	Transfer top layer to a labeled microcon assembly. Appropriately discard the tube containing the bottom layer.	
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If phase lock tubes are not used, be careful not to pipette the bottom layer or the interface between the layers.

If multiple extracts are to be combined (e.g., multiple swabs or cuttings extracted separately, to include those previously extracted), add only one extract to the microcon at a time and spin prior to adding subsequent extract to the microcon. Steps 4.3.4 through 4.3.6 will be repeated, as necessary, for those extracts and corresponding reagent blanks being combined.

4.3.5	Spin labeled the microcon assemblies (generally between 6,000 and 8,000 rpm for 10 minutes).	
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Additional spins may be used to draw fluid through the membrane. Speed and/or time may be increased, but excess speed and/or time should be avoided to prevent damaging the membrane.

4.3.6	Discard waste. (<i>By decanting or pipetting, entire waste volume does not need to be removed.</i>)	
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4.3.7	Add 200 µL reagent grade water to each microcon.	
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4.3.8	Spin (generally between 6,000 and 8,000 rpm for 10 minutes).	
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Additional spins may be used to draw fluid through the membrane. Speed and/or time may be increased, but excess speed and/or time should be avoided to prevent damaging the membrane.

If needed, additional reagent grade water washes (steps 4.3.6 through 4.3.8) may be performed and must be carried out in parallel on the corresponding reagent blank(s).

If additional spins or washes do not reduce the volume, the affected sample(s) may continue with

processing at step 4.3.9. Record the final volume.

4.3.9	Add reagent grade water (generally 15 µL) to each microcon. Invert each microcon into a new, labeled tube. Spin (generally between 9,000 and 13,000 rpm for 5 minutes).	
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4.3.10	Ensure final tubes are barcoded.	
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If the final extract displays discoloration, a dilution may be prepared with reagent grade water.

Refer to the appropriate DNA procedure (i.e., DNA 226 or DNA 232) if samples need to be combined/concentrated following extraction.

6 Sampling or Sample Selection

Not applicable.

7 Calculations

Not applicable.

8 Measurement Uncertainty

Not applicable.

9 Limitations

9.1 The quantity and quality of the DNA present within any biological material ultimately determines if a DNA extraction is successful.

10 Safety

10.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

10.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

10.3 Procedural Specific Chemical Hazards:

- Solutions of Proteinase K can be irritating to mucous membranes. Use eye protection when handling.
- PCIA (Phenol/Chloroform/Isoamyl Alcohol) can cause burns and is toxic by inhalation, contact with skin, and if swallowed. Its use will be confined to a fume hood.

11 References

FBI Laboratory Quality Assurance Manual

FBI Laboratory Operations Manual

FBI Laboratory Safety Manual

DNA Procedures Manual

5 Prime Manual Phase Lock Gel™ (PLG) User Guide, 2007.

Comey CT, Koons BW, Presley KW, Smerick JB, Sobieralski CA, Stanley DM, and Baechtel FS. DNA extraction strategies for amplified fragment length polymorphism analysis. *Journal of Forensic Sciences* (1994) 39: 1254-1269.

Millipore Corporation. *Microcon® Centrifugal Filter Devices User Guide*. Millipore Corporation, Billerica, MA, 2013. (Available at http://www.emdmillipore.com/Web-US-Site/en_CA/-/USD/ShowDocument-File?DocumentId=201306.3829.ProNet&ProductSKU=MM_NF-MRCF0R100&Language=EN&DocumentType=UG&Origin=PDP&Country=NF)

Qiagen® *MaXtract Low and High Density Handbook*, October 2006.

Rev. #	Issue Date	History
1	07/09/13	<p>2: Reagents section moved to sections 4.1 and 4.2 for the two extraction procedures. Refers the user to those sections.</p> <p>4.1: Removed TE buffer; added reagent grade water. Removed “prepared aliquot” from the Proteinase K description.</p> <p>After 4.1.6 and after 4.2.14: Disposal of PCI and PCI-contaminated consumables in an appropriate waste container is required.</p> <p>4.1.12, after 4.1.13, and 4.1.14: Replaced TE with reagent grade water.</p> <p>4.1.14: Changed the usual volume from 25 µL to 15 µL, added 4.1.15 to this step; deleted 4.1.15 and renumbered.</p> <p>After 4.1.16: Replaced TE with reagent grade water for dilutions. Added reference to SOP 222 for combining/concentrating.</p> <p>4.2: New section added that describes the differential extraction procedure, previously in DNA SOP 202-8. Renumbered remaining sections. Procedure reflects previous procedure with the following exceptions:</p> <p>4.2.3: Decreased the volume of master mix added to each tube from 500 µL to 450 µL.</p> <p>4.2.11: Increased the reagent volumes for master mix.</p> <p>4.2.12: Increased the volume of master mix added to each tube from 360 µL to 450 µL.</p> <p>4.2.14: Increased the volume of PCI added to each tube from 400 µL to 450 µL.</p> <p>4.2.20, after 4.2.21, and 4.2.22: Replaced TE with reagent grade water.</p> <p>4.2.22: Changed the usual volume from 25 µL to 15 µL.</p> <p>After 4.2.25: Replaced TE with reagent grade water for dilutions. Added reference to SOP 222 for combining/concentrating.</p> <p>4.3: Removed combining/concentrating section and referred the user to SOP 222.</p>
2	02/28/18	<p>Adjusted scope to apply to DNA personnel.</p> <p>Relocated reagent lists to section 2.</p> <p>Updated DNA procedure references throughout.</p> <p>Changed all PCI abbreviations to PCIA.</p> <p>Consolidated PCIA and Microcon procedures to 4.3. Renamed prior sections.</p>

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 02/27/2018

DCU Chief

Date: 02/27/2018

BAU Chief

Date: 02/27/2018

QA Approval

Quality Manager

Date: 02/27/2018

Procedures for the Semi-Automated Extraction of DNA

1 Scope

These procedures apply to DNA personnel performing semi-automated extraction and purification of deoxyribonucleic acid (DNA) using the QIAcube[®], EZ1[®] Advanced XL (EZ1[®]), and/or QIASymphony[®] SP in the DNA Casework Unit (DCU) or Biometrics Analysis Unit (BAU) and DNA personnel that perform the associated quality control procedures.

2 Equipment/Materials/Reagents

Materials

- General laboratory supplies (e.g., tubes, pipettes)
- Costar[®] spin baskets, or equivalent (*for differential extractions*)
- Qiagen[®] Lyse & Spin Baskets and Collection Tubes, or equivalent (*for normal extractions*)
- QIAcube[®]
- QIAcube[®] consumables (e.g., 1000 µL wide-bore filter-tips, reagent bottles, rotor adapters, QIAcube[®]-compatible 1.5 mL microcentrifuge tubes)
- EZ1[®] Advanced XL
- EZ1[®] DNA Investigator Kit (e.g., filter tips, tip holders, elution tubes, reagent cartridges)
- QIASymphony[®] SP
- QIASymphony[®] DNA Investigator Kit (e.g., reagent cartridges, piercing lid, enzyme rack, Reuse Seal Strips)
- QIASymphony[®] SP consumables (8-Rod Covers, Sample Prep Cartridges, 200 µL tips, and 1500 µL tips)
- Speed-Vac, Vacufuge Concentrators, or equivalent

Reagents

- Buffer ATL
- Buffer G2 (*for EZ1 normal lysis and EZ1 male fraction only*)
- 1M Dithiothreitol (DTT)
- Proteinase K
- Reagent Grade Water

3 Standards and Controls

At least one extraction control (i.e., reagent blank) must be processed in parallel with each extraction batch.

For evaluation of the extraction controls, refer to the appropriate interpretation procedure of the *DNA Procedures Manual*.

4 Procedures

Refer to the DNA Procedures Introduction (DNA QA 600) for applicable general precautions and cleaning instructions.

Ensure the appropriate fields (i.e., instruments, reagents) in the Sample Tracking and Control Software (STACS) are completed from any network computer, as necessary.

4.1 Normal Lysis

4.1.1	Create master mix using the appropriate recipe listed below.	
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If precipitate has formed in the Buffer ATL, heat it, generally at 56°C, until precipitate is no longer visible.

Normal Lysis Master Mix

EZ1® Advanced XL

Reagent	μL per sample
Buffer G2	470
Pro K	15
DTT	15

QIAasympohony®

Reagent	μL per sample
Buffer ATL	460
Pro K	20
DTT	20

The samples should be in Lyse & Spin baskets in corresponding tubes.

4.1.2	Add 450 μL master mix.	
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4.1.3	Incubate the tubes in an incubator at 56°C with agitation (generally 200 rpm) for ~1 hour.	
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4.1.4	Spin the tubes (generally between 9,000 and 13,000 rpm for 5 minutes). Discard the basket.	
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If the lysate does not completely flow through the basket, additional spins may be added. If necessary, lysate remaining in the basket may be manually transferred to the sample tube. Additional manipulations will be recorded in the case notes. If the volume in the sample tube is significantly different than the expected volume a case note will be made.

4.1.5	Process the lysates on the EZ1® following the steps in section 4.3 or QIAasympohony® SP following the steps in section 4.4, as appropriate.	
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4.2 Differential Lysis

4.2.1	Create the epithelial (F) fraction master mix.	
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If precipitate has formed in the Buffer ATL, heat it, generally at 56°C, until precipitate is no longer visible.

Female Fraction Lysis Master Mix

Reagent	μL per sample
Buffer ATL	160
Reagent Grade Water	320
Pro K	20

The samples should be in QIAcube® compatible tubes. Lyse & Spin baskets must NOT be used for differential extractions.

4.2.2	Add 450 μL master mix.	
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4.2.3	Incubate the tubes at 56°C with agitation (generally 900 rpm) for ~1.5 hours.	
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Generally, differential incubations are done in a thermomixer.

4.2.4	If necessary, quick spin and transfer the substrate to a basket. Spin the tubes (generally between 9,000 and 13,000 rpm for 5 minutes). Discard the basket.	
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4.2.5	Ensure consumables, reagent grade water, and lysate tubes are properly loaded onto the QIAcube® instrument.	
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Consumables include: bottles containing reagent grade water, 1000 μL wide-bore tips, rotor adapters, and F fraction collection tubes. A shaker rack plug should also be in place next to each F fraction collection tube.

Appendix A has additional guidance for loading the QIAcube®.

4.2.6	Initiate the “Separate and Lyse 12A Mod” protocol on the QIAcube®.	
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The bottle in position 1 will contain reagent grade water, not Buffer G2 as prompted. Be aware that lysates are 450 μL, not 500 μL as referenced in the QIAcube® prompt.

4.2.7	At the completion of the run, tubes containing the F fraction should be removed and capped. Refill consumables and reagent grade water as necessary.	
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The sperm (M) fractions continue processing on the QIAcube®.

Processing of the F fraction resumes at section 4.3, either independently or with the M fraction.

4.2.8	Initiate the “Separate and Lyse 12B Mod” protocol on the QIAcube®.	
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The bottles will contain reagent grade water, not Buffer G2 as prompted.

4.2.9	At the completion of the run, remove and cap the tubes containing the M fraction(s).	
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4.2.10	A slide for microscopy may be prepared from the M fraction according to Serology Procedure 112 (Sero 112).	
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4.2.11	Ensure the M fraction master mix is created.	
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Male Fraction Lysis Master Mix

EZ1® Advanced XL

Reagent	µL per sample
Buffer G2	375
Pro K	24
DTT	94

QIAasymphony®

Reagent	µL per sample
Buffer ATL	375
Pro K	24
DTT	94

4.2.12	Add 450 µL master mix to each M fraction tube.	
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4.2.13	Vortex, quick spin, and incubate the tubes at 70°C with agitation (generally 900 rpm) for ~10 minutes.	
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4.2.14	Vortex the tubes vigorously (~10 seconds) and quick spin.	
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4.2.15	Process the lysates on the EZ1® following the steps in section 4.3 or QIAasymphony® SP following the steps in section 4.4, as appropriate.	
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Ensure waste is disposed of properly.

The water bottles on the QIAcube® should be emptied at the end of each day of use.

4.3 Processing Lysates on the EZ1® Advanced XL

Lysates may be stored refrigerated for up to 6 days prior to processing on the EZ1®. Ensure that no precipitate remains in the lysates prior to processing.

4.3.1	Ensure the EZ1® is UV irradiated for 20 minutes prior to initial use each day.	
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If precipitate has formed in the EZ1[®] cartridges, heat them, generally at 56°C, until precipitate is no longer visible.

If necessary, transfer the lysate to an EZ1[®] compatible tube.

4.3.2	If appropriate, two lysates may be combined prior to loading on the EZ1 [®] .	
4.3.3	Ensure consumables, lysate tubes, and barcoded elution tubes are properly loaded onto the EZ1 [®] . See Figure 1.	

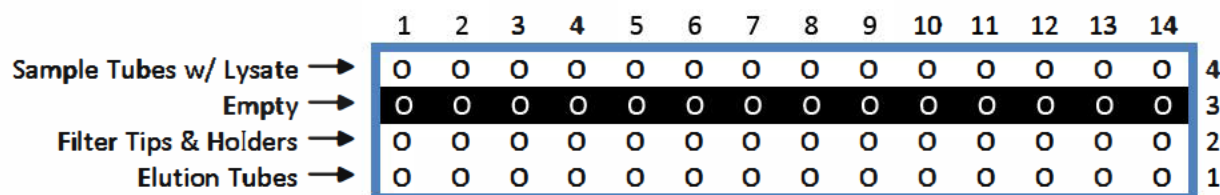


Figure 1 - Loading the EZ1[®] Advanced XL

4.3.4	Ensure the Large Volume protocol with elution into 50 µL of water has been selected and start the EZ1 [®] .	
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Disregard the reference to MTL Buffer in the prompts on the EZ1[®].

4.3.5	At the completion of the run, remove and cap elution tubes.	
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If the volume in the elution tube is significantly different than the expected volume a case note will be made.

4.3.6	If necessary, combine appropriate extracts.	
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If combined samples will be concentrated using the Speed-Vac, a maximum of eight 50 µL extracts may be combined into one tube.

4.3.7	Ensure waste is disposed of properly. Ensure the piercing units on the EZ1 [®] are cleaned after daily use and clean the tray, worktable, and racks, if needed.	
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Bleach should not be used on the QIAcube[®] or the EZ1[®] Advanced XL. 70% ethanol should be used to clean instrument surfaces.

4.4 Processing Lysates on the QIAsymphony[®] SP

Lysates may be stored refrigerated for up to 6 days prior to processing on the QIAsymphony[®] SP. Ensure that no precipitate remains in the lysates prior to processing.

4.4.1	Remove the waste chute and tip park station. Ensure the QIA Symphony® SP deck is empty prior to UV irradiation and that the instrument is UV irradiated for at least 15 minutes prior to initial use each day.	
4.4.2	Ensure the QIA Symphony® SP is prepared for the run. <ul style="list-style-type: none"> • Load consumables, waste chute, tip park station, sample tubes containing lysate, and barcoded elution tubes. • Select the Custom Protocol. • If necessary, change the tube type.* • Select the appropriate elution volume, generally 100 µL. • Associate the appropriate sample racks with the corresponding elution tube racks. 	

** If the tube volume default is 2 mL, the setting for any position in the sample rack that contains a 1.5 mL tube (e.g., QIAcube® tube) should be changed to reflect the appropriate tube volume.*

Appendix B has additional guidance for loading the QIA Symphony® SP.

4.4.3	Start the QIA Symphony® SP.	
4.4.4	At the completion of the run, remove and cap elution tubes. Verify the volume in the elution tubes prior to discarding the components on the deck.	

Empty sample tubes may be discarded after processing of the sample rack. If significant volume remains in a sample tube, it should be retained until elution volume is verified.

If eluate is not present in an elution tube, the QIA Symphony® SP User Manuals may be referenced for troubleshooting and potential sample recovery procedures.

4.4.5	If necessary, combine appropriate extracts.	
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If combined samples will be concentrated using the Speed-Vac, a maximum of four 100 µL extracts may be combined into one tube.

Ensure waste is properly disposed (i.e., lysis tubes, tip waste, and liquid waste).

All plastics and reagents should be removed following daily use. The reagent cartridge should be capped prior to storage or disposal.

The deck, back tray, magnetic head guards, tip guards, tip park station, and waste chute should be cleaned following daily use with 70% isopropanol (use water, not alcohol, on the hood of the instrument).

Bleach should not be used on the QIA Symphony® SP.

Refer to the QIAcube® User Manual and the QIAsymphony®SP User Manuals for guidance on instrument maintenance and deeper cleaning, as required.

4.5 Concentrating Extracted Samples Using the Speed-Vac or Vacufuge

The Speed-Vac flask should be empty and dry, and the flask seal should be tight.

The Speed-Vac should be turned on ~45 minutes prior to use.

Ensure the gasket on the centrifuge is in its proper position and that the rotor is properly tightened during sample processing.

4.5.1	Samples from questioned items (and corresponding reagent blanks) are typically concentrated using a Speed-Vac or Vacufuge and reconstituted with 15 µL with reagent grade water.	
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If an alternate volume of reagent grade water is used to reconstitute, it will be documented.

Samples from known items (and corresponding reagent blanks) typically do not require concentration.

On the Speed-Vac with the heat set to “High”, a 50 µL extract may take ~30-40 minutes to dry and a 100 µL extract may take ~60 minutes to dry. Samples should not be dried on “High” for more than four hours (maximum starting volume of ~400 µL).

On the Vacufuge with a setting of 60°C, a 50 µL extract takes about 45 minutes.

Alternatively, combining/concentrating can be done using a Microcon Filter rather than a Speed-Vac.

4.6 Combining/Concentrating Extracted Samples Using a Microcon Filter

4.6.1	Vortex and quick spin the extract tubes. Transfer the extract for each sample being combined/concentrated into a labeled microcon assembly.	
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Corresponding reagent blanks must also be microconned.

4.6.2	Spin the tubes to draw the fluid through the membrane (generally between 6,000 and 8,000 rpm for 10 minutes).	
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Speed and time may be increased to draw fluid through membrane, but to avoid damage to the membrane, excess speed and time should not be used.

If additional spins do not reduce the volume, the affected sample(s) may continue with processing at step 4.6.4. Record the final volume.

4.6.3	Add reagent grade water (generally 15 µL).	
4.6.4	Invert microcon into a new, labeled tube.	
4.6.5	Spin the tubes (generally between 9,000 and 13,000 rpm for 5 minutes).	
4.6.6	Ensure the final tubes are barcoded.	

5 Sampling or Sample Selection

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

The quantity and quality of the DNA present within any biological material ultimately determines if a DNA extraction is successful.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Procedural Specific Chemical Hazards:

- Solutions of Proteinase K can be irritating to mucous membranes. Use eye protection when handling.

- EZ1[®] reagent cartridges, QIAasymphony[®] reagent cartridges, and QIAasymphony[®] liquid waste contain ethyl alcohol and guanidine salts which are hazardous materials. Solutions containing guanidine salts will generate toxic fumes when combined with bleach. Use appropriate care and wear appropriate protective clothing and eyewear when handling. Be careful not to expose face or hands to splashes. Dispose of EZ1[®] and QIAasymphony[®] consumables in appropriate waste containers.

10 References

FBI Laboratory Safety Manual

DNA Procedures Manual

Qiagen[®]. *QIAcube[®] User Manual*, June 2008.

Qiagen[®]. *EZ1[®] Advanced XL User Manual*. May 2009.

Qiagen[®]. *EZ1[®] DNA Investigator Handbook*. April 2009.

Qiagen[®]. *QIAasymphony[®] DNA Investigator Handbook*. February 2013.

Qiagen[®]. *QIAasymphony[®] SP/AS User Manual – General Description*. May 2013.

Qiagen[®]. *QIAasymphony[®] SP/AS User Manual – Operating the QIAasymphony[®] SP*. April 2012.

Qiagen[®]. *QIAasymphony[®] SP/AS Management Console User Manual (software version 4.0)*. April 2012.

Qiagen[®]. *QIAasymphony[®] SP/AS Consolidated Operating Guide*. May 2013.

Rev. #	Issue Date	History
2	02/28/18	Revised scope. Added BAU. Updated references throughout. Added general introduction information to procedures. 4.3.7: Added EZ1 cleaning guidance in lieu of referring to user manual. Added Appendix C with instrument QC procedures from STACS instructions.
3	02/15/19	2 & 4.5: Added Vacufuge concentrator in addition to Speed vac and default settings. 4.1.4 & 4.3.5: Added allowance for additional spins and instruction to note any volume discrepancies after spins or elution. Appendix C: Changed QC samples to swabs.

Approval

Redacted - Signatures on File

DNA Technical Leader Date: 02/14/2019

Acting BAU Chief Date: 02/14/2019

DCU Chief Date: 02/14/2019

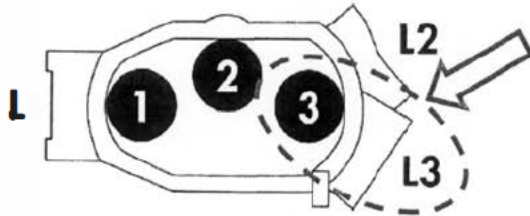
DSU Chief Date: 02/14/2019

QA Approval

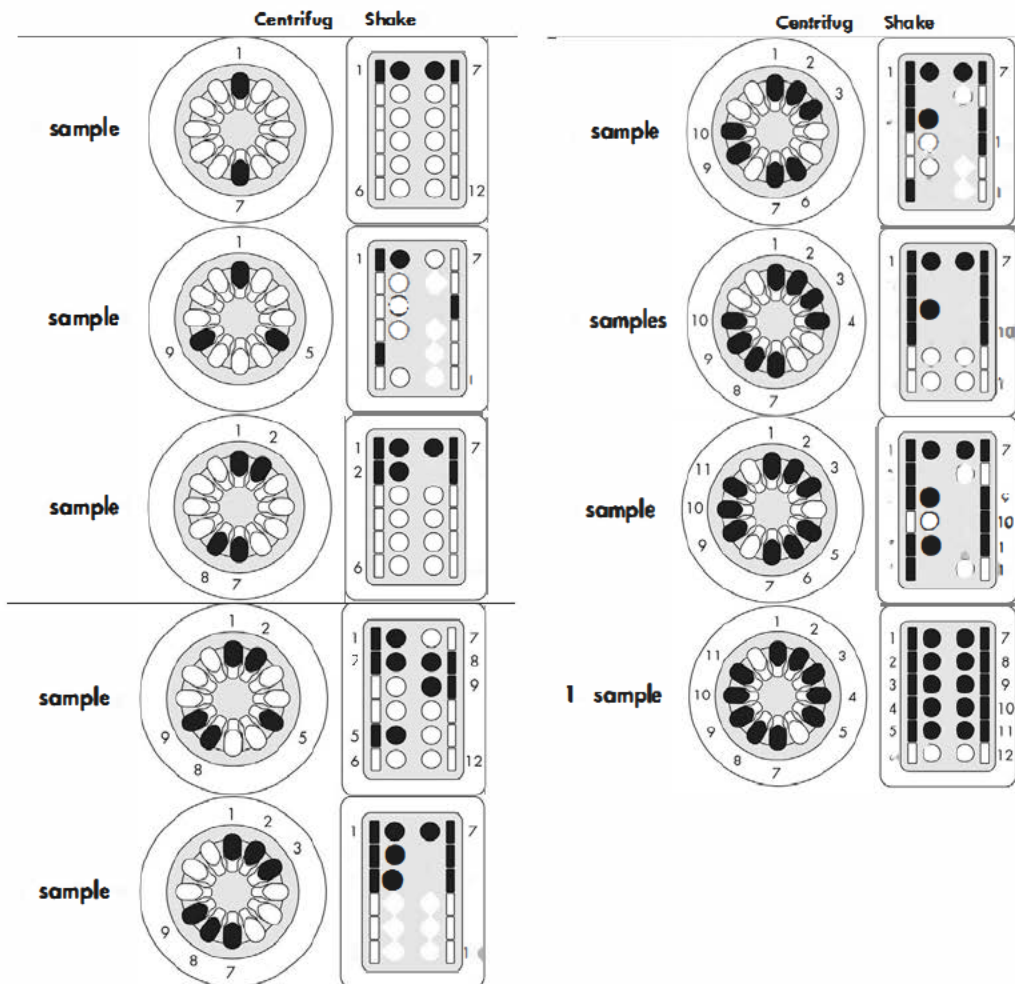
Quality Manager Date: 02/14/2019

Appendix A Loading the QIAcube

The lysate tube fits into position 1 of the rotor adaptors with the cap folded back and inserted into position L3.



Rotor adaptor with lysate tube must be distributed on the centrifuge for balance.



Appendix A: Loading the QIAcube® (continued)

An overview of the loaded QIAcube®.



Appendix B: Loading the QIAsymphony®SP

The QIAsymphony®SP is comprised of four drawers that can be loaded in any order.

A wizard is available from the main screen to assist in loading.

A. Sample drawer

Load barcoded tubes containing lysate into a sample rack(s), and then slide the sample rack(s) into the appropriate position(s) in the sample drawer.



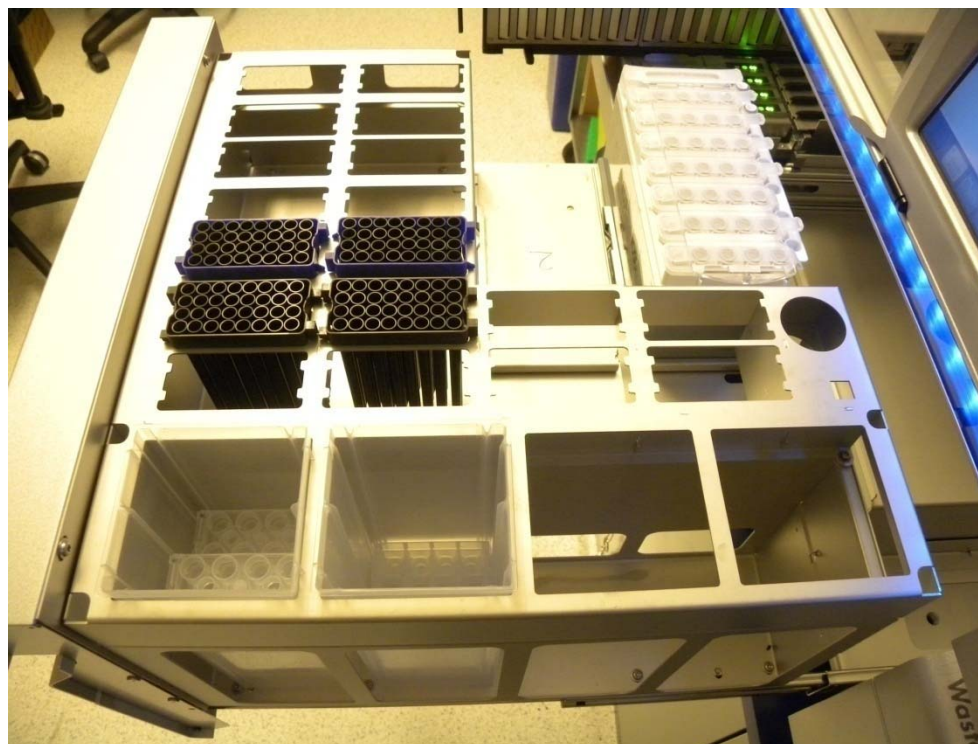
Appendix B: Loading the *QIA*symphony® SP (continued)

B. Reagents and Consumables drawer

Load sufficient reagents and consumables to process the appropriate number of samples. This drawer will contain reagent cartridge(s), 200 μ L tips, 1500 μ L tips, sample prep cartridge(s), and 8-rod covers.

Reagent Cartridge preparation (in any order):

- Attach enzyme rack to the reagent cartridge.
- Add ~1600 μ L of reagent grade water to both tubes in the enzyme rack.
- Place the reagent cartridge in the reagent cartridge holder.
- Remove the magnetic bead trough and vortex or shake for at least one minute. Replace the trough in the proper orientation. If first use, remove the foil cover. For subsequent uses, remove the Reuse SealStrip.
- If first use, place the piercing lid on top of the reagent cartridge. For subsequent uses, remove the reagent cartridge Reuse SealStrips prior to loading.

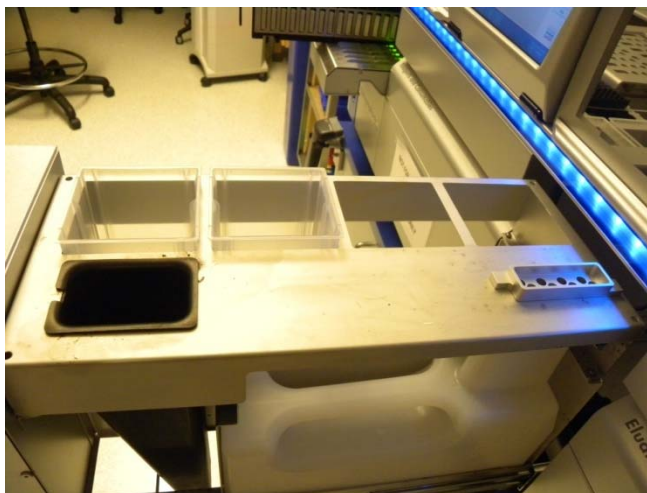


Appendix B: Loading the QIAasymphony® SP (continued)

C. Waste drawer

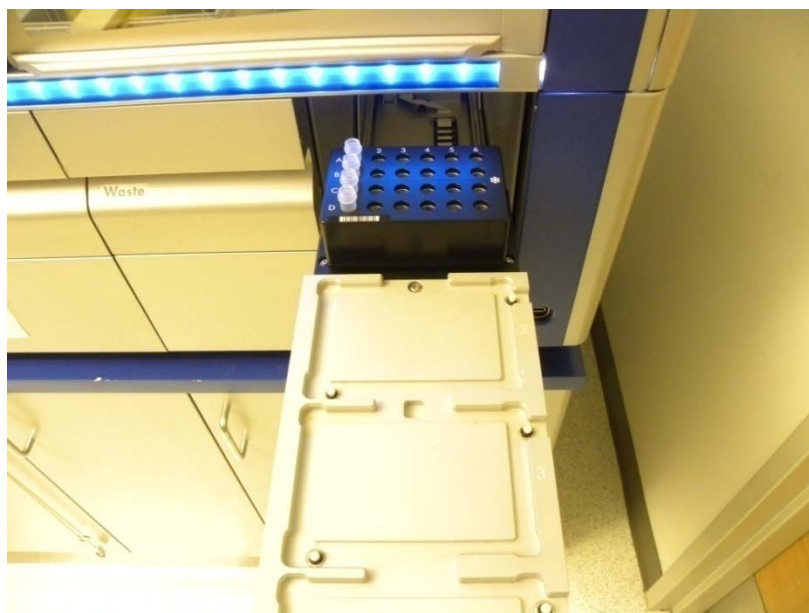
Ensure plastic vessels (empty 8-rod cover or sample prep cartridge containers), the waste chute, the liquid waste jug, and the tip park station are in place.

Ensure that the tip waste container in bottom right cabinet is not completely full.



D. Eluate drawer

Barcoded elution tubes should be loaded into an elution rack. The number and order of tubes loaded should correspond to the number and order of tubes containing lysate loaded in the sample drawer. The elution rack(s) are loaded back-to-front in column format.



Appendix C: Instrument General Maintenance and Performance Verification Procedures

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency of performance verifications and additional requirements.

1. QIAcube® Robotic System (Extraction)

A. General Maintenance

There is no general maintenance required for the QIAcube®.

B. Performance Verification

A portion of a swab containing 5 µL female blood and 1 µL of a 10x dilution of semen (semen diluted with reagent grade water) with known typing results will be processed using the QIAcube® differential lysis procedure followed by extraction using the appropriate DNA standard operating procedure (SOP).

The mixture must yield a distinguishable M fraction containing predominantly male DNA with minimal carryover from the F fraction as determined by quantification and amplification. The mixture is expected to yield a distinguishable F fraction containing predominantly female DNA but may contain a mixture of male and female DNA depending on the sample.

If the sample does not produce a distinguishable M fraction the process should be repeated with a new sample. If a predominately male DNA containing M fraction is not accomplished after 2 attempts the Technical Leader (TL) will be consulted.

2. EZ1® Advanced XL Robotic System (Extraction)

A. General Maintenance

Quarterly: Inspect the O-rings and grease as needed.
Test Heating Block at 70°C.

B. Performance Verification

A swab containing 5 µL blood from a known donor will be processed using the Normal lysis procedure followed by the EZ1® extraction procedure found in the appropriate DNA SOP for each of the 14 channels of the EZ1 robot. Alternately, individual channels may be tested to verify performance as needed.

A sample from each channel must yield a concentration, determined by quantification, greater than the minimum value established for the specific donor batch on the EZ1® using a 50 µL elution volume. A channel, or channels, may be repeated if necessary.

If a channel, or channels, does not yield the appropriate concentration after 2 attempts the TL will be consulted.

3. QIAsymphony® SP Robotic System (Extraction)

A. General Maintenance

Monthly or at 1000 runs: Change O-rings

B. Performance Verification

A swab containing 5 µL blood from a known donor will be processed using the Normal lysis procedure followed by the QIAsymphony® extraction procedure found in the appropriate DNA SOP for each of the 4 channels of the QIAsymphony® instrument. Alternately, individual channels may be tested to verify performance as needed.

A sample from each channel must yield a concentration, determined by quantification, greater than the minimum value established for the specific donor batch on the QIAsymphony® using a 100 µL elution volume without TopElute fluid (TOPE). A channel, or channels, may be repeated if necessary.

If a channel, or channels, does not yield the appropriate concentration after 2 attempts the TL will be consulted.

4. Establishing the minimum value for Extraction Robot PV

Use an in service instrument and the appropriate extraction procedure to extract several samples from a blood card (i.e., FTA card) or swabs spotted with donor blood. The minimum quantitation value will be established by calculating the average quantitation of the extracted samples minus three standard deviations.

Procedures for the Interpretation of GlobalFiler™ and Identifiler® Plus Data

1 Scope

These procedures apply to DNA personnel who verify and interpret nuclear DNA typing results obtained from the GlobalFiler™ and/or Identifiler® Plus PCR Amplification Kit using the GeneMapper® ID-X (GMIDX) DNA typing software for forensic comparison purposes and perform interpretation and statistical analysis using STRmix™.

2 Background

Upon completion of the technical aspects of nuclear DNA analysis, the results must be verified and interpreted by an Examiner. The verification of the accuracy of the results involves a review of peak designations and other information generated by the appropriate DNA typing software, as well as an evaluation of quality controls. Following this assessment, the Examiner makes comparisons among samples and draws conclusions that are captured for documentation and communication purposes within an FBI *Laboratory Report* (7-1, 7-1 LIMS, 7-273, or 7-273 LIMS).

The results are derived through application of the appropriate software during and after capillary electrophoresis (CE) of amplified DNA that is generated for each specimen using the GlobalFiler™ Amplification Kit with 28 cycles of PCR for all sample types. The number of contributors to the DNA typing results is determined, and based on a visual comparison of the DNA typing results, the Examiner may conclude that a person of interest (POI) is excluded as a possible contributor. DNA profiles from which a POI cannot be visually excluded as a possible contributor may be imported into the STRmix™ software.

STRmix™ calculates a likelihood ratio (LR) that reflects the probability of the DNA typing results under two opposing hypotheses: H_1 , which includes the POI as a contributor to the evidence, and H_2 , which does not include the POI as a possible contributor. These hypotheses typically align with the prosecution and defense positions, respectively, and as such are sometimes referred to as the prosecution hypothesis (H_p) and the defense hypothesis (H_d). The value of the LR leads to the conclusions that are captured within a written report.

The reinterpretation of legacy data from the Identifiler® Plus PCR Amplification Kit will follow these procedures and reporting language with supplemental information specific to the Identifiler® Plus data noted in Appendix E. For interpretation of legacy STR amplification kit data using a statistical approach other than STRmix™ (i.e., random match probability, combined probability of inclusion, or kinship analysis), refer to the procedures for Interpretation of Legacy DNA Data (i.e., DNA 230).

Familial comparisons of DNA results will be conducted using the appropriate interpretation protocol of the *DNA Procedures Manual* (i.e., DNA 227).

3 Equipment/Materials/Reagents

GeneMapper® ID-X Software (Applied Biosystems, version 1.6 or higher)

STRmix™ (NicheVision Forensics LLC, version 2.4 or higher)

4 Standards and Controls

Raw data for the electrophoretic runs of samples or controls displaying no typing results must be reviewed for the presence of a primer peak. If no primer peak is observed, the sample must be reinjected or reprepared to verify that amplicon was added to the CE plate.

4.1 Verification of GeneScan™ 600 LIZ v2.0 Internal Size Standard (GS-600v2)

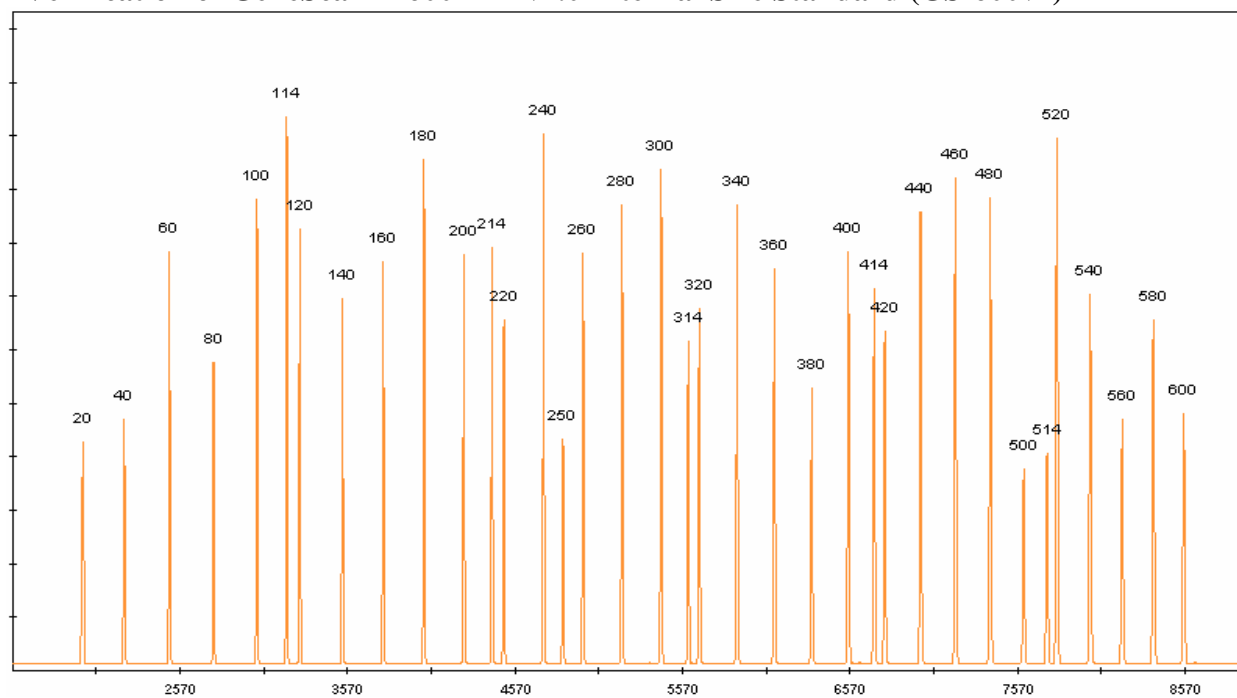


Figure 1 – GS-600v2 Internal Size Standard Peak Series

4.1.1 For GlobalFiler™ data, which is sized using the Local Southern Method, verify that the 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, and 460 base pair (bp) fragments of the GS-600v2 (Figure 1) are captured and have been assigned the correct size values for each sample, control, and allelic ladder. Fragments 60 through 460 must be captured and assigned the correct size values for a given injection of a sample to be interpreted. Note: Due to the temperature sensitivity of the 250 bp fragment's sequence-based conformation, this fragment is not used for sizing purposes.

4.1.2 If all of the GS-600v2 fragments for a given injection of a sample do not meet these specifications, a different injection of the sample that does display the correct size values for all of the GS-600v2 fragments must be used for interpretation of the entire DNA profile, which may require that the sample be reprocessed.

4.2 Verification of Allelic Ladders

Locus	Known Size Range (bp) ¹	Alleles Present in Ladder ²	Color
D3S1358	96 - 142	9 - 20	Blue
vWA	156 - 209	11 - 24	Blue
D16S539	227 - 268	5, 8 - 15	Blue
CSF1PO	283 - 319	6 - 15	Blue
TPOX	338 - 379	5 - 15	Blue
Y indel	81 - 86	1, 2	Green
Amelogenin	98 - 105	X, Y	Green
D8S1179	114 - 171	5 - 19	Green
D21S11	183 - 240	24, 24.2, 25, 26, 27, 28, 28.2, 29, 29.2, 30, 30.2, 31, 31.2, 32, 32.2, 33, 33.2, 34, 34.2, 35, 35.2, 36, 37, 38	Green
D18S51	261 - 343	7, 9, 10, 10.2, 11, 12, 13, 13.2, 14, 14.2, 15 - 27	Green
DYS391	365 - 389	7 - 13	Green
D2S441	76 - 114	8 - 11, 11.3, 12 - 17	Yellow
D19S433	118 - 172	6 - 12, 12.2, 13, 13.2, 14, 14.2, 15, 15.2, 16, 16.2, 17, 17.2, 18.2, 19.2	Yellow
TH01	179 - 218	4 - 9, 9.3, 10, 11, 13.3	Yellow
FGA	223 - 379	13 - 26, 26.2, 27 - 30, 30.2, 31.2, 32.2, 33.2, 42.2, 43.2, 44.2, 45.2, 46.2, 47.2, 48.2, 50.2, 51.2	Yellow
D22S1045	88 - 121	8 - 19	Red
D5S818	138 - 183	7 - 18	Red
D13S317	199 - 243	5 - 16	Red
D7S820	262 - 299	6 - 15	Red
SE33	307 - 429	4.2, 6.3, 8, 9, 11 - 20, 20.2, 21, 21.2, 22.2, 23.2, 24.2, 25.2, 26.2, 27.2, 28.2, 29.2, 30.2, 31.2, 32.2, 33.2, 34.2, 35, 35.2, 36, 37	Red
D10S1248	85 - 130	8 - 19	Purple
D1S1656	160 - 207	9 - 14, 14.3, 15, 15.3, 16, 16.3, 17, 17.3, 18.3, 19.3, 20.3	Purple
D12S391	216 - 269	14 - 19, 19.3, 20 - 27	Purple
D2S1338	281 - 350	11 - 28	Purple

Table 1 – GlobalFiler™ Allelic Ladder Specifications

¹ Sizes are based on Applied Biosystems' precision studies of the GlobalFiler™ Allelic Ladder published in the *GlobalFiler™ User Guide*. Sizes may vary due to electrophoretic effects.

² Unless otherwise indicated, ranges of alleles (e.g., 12 - 19) include only integers (e.g., 12, 13, 14, ..., 19).

4.2.1 Any allelic ladder used for genotyping must: 1) exhibit the correct allele designations (see Table 1) and 2) yield the correct typing results when used to genotype the positive amplification control.

4.2.2 If any sample(s) requires reinjection, the appropriate ladder must be included in the reinjection set.

4.3 Positive Amplification Control (i.e., 007)

4.3.1 One positive control must be processed in parallel with each amplification set or batch of samples.

4.3.1.1 If any sample(s) requires reparation, the positive control and the appropriate ladder must also be reprepared.

4.3.2 If a set of samples has multiple injections of the positive control, at least one injection must exhibit all the expected allelic peaks ≥ 150 relative fluorescence units (RFU), and must not exhibit any extraneous allelic peaks. A positive control with a non-allelic peak(s) (e.g., stutter, spike, pull-up) may be interpreted. See Table 2 for the expected positive control typing results obtained using GlobalFiler™.

Locus	007
D3S1358	15, 16
vWA	14, 16
D16S539	9, 10
CSF1PO	11, 12
TPOX	8
Y indel	2
Amelogenin	X, Y
D8S1179	12, 13
D21S11	28, 31
D18S51	12, 15
DYS391	11
D2S441	14, 15
D19S433	14, 15
TH01	7, 9.3
FGA	24, 26
D22S1045	11, 16
D5S818	11
D13S317	11
D7S820	7, 12
SE33	17, 25.2
D10S1248	12, 15
D1S1656	13, 16
D12S391	18, 19
D2S1338	20, 23

Table 2 – Expected DNA Typing Results of the Positive Control for STR Loci Analyzed Using the GlobalFiler™ Kit

4.3.2.1 If the positive control does not exhibit the expected results, one or more of the following actions must be taken:

- a. The positive control may be regenotyped using an alternate ladder(s) that was run in parallel with the positive control. If this positive control then exhibits the expected results, any samples that are successfully genotyped using the alternate ladder may be interpreted.
- b. If regenotyping of the positive control does not result in the expected results, the positive control may be reinjected using the same CE daughter plate together with a ladder. If the positive control then exhibits the expected genotype, all samples may be interpreted using the ladder(s) that produces the expected positive control genotype.
- c. Alternatively, the positive control may be reprepared onto a new CE daughter plate together with a ladder and all samples that were injected in parallel. If the positive control displays the expected genotypes, all samples that were injected from the reprepared CE daughter plate may be interpreted.
- d. If additional allelic peaks are present in the positive control (i.e., it is contaminated), then all samples amplified in parallel with this control should be assessed as described in section 5.2.
- e. If after reinjection and/or repreparation, the results indicate that a DNA source other than the positive control was substituted for this control DNA, or if the positive control displays no results, then any samples amplified in parallel with this control may not be interpreted.

4.4 Negative Amplification Control

A negative control must be processed in parallel with each amplification set or batch of samples. If the negative control does not result in the expected results, one or more of the following actions must be taken:

- a. If at least one extraction control or negative sample on the original plate is clean, the negative control may be reprepared onto a new CE daughter plate together with a ladder and the positive control. If the negative control displays the expected results, all samples may be interpreted.
- b. Alternatively, if there are no clean extraction controls or negative samples on the original plate, the entire plate may be reprepared onto a new CE daughter plate. If the negative control displays the expected results, all reprepared samples may be interpreted.
- c. If the negative control still does not display the expected results, see sections 5.1.1.1 (Identification of Peaks of Non-Genetic Origin) or 5.2 (Contamination) for guidance on how to treat unresolved peaks observed in the negative control.

4.5 Extraction Control (i.e., Reagent Blank)

4.5.1 At least one extraction control must be processed in parallel with each batch of samples subjected to a specific extraction process.

4.5.2 If a sample needs to be reamplified or reinjected, and the extraction control processed in parallel with this sample does not have any typing results, then the extraction control does not need to be reprocessed.

See sections 5.1.1.1 (Identification of Peaks of Non-Genetic Origin) or 5.2 (Contamination) for guidance on how to treat peaks observed in extraction controls.

5 Procedures

5.1 DNA Profile Determination

5.1.1 Computer Assisted Allele Designations

The GMIDX software, using the analysis method settings for GlobalFiler™ data represented in Appendix A, analyzes the data generated by the CE instruments and generates electropherogram data to be evaluated and interpreted. The analysis method settings for Identifiler® Plus data are contained in the procedure for interpretation of results from the Identifiler® Plus amplification kit (i.e., DNA 229) and/or the procedures for interpretation of legacy DNA data (i.e., DNA 230). A pink box surrounding a data point label indicates that the software has identified a data point as an artifact. The GMIDX software uses the terms “spike” and “OMR” (Outside Marker Range) to represent a variety of DNA artifacts. Peak labels may be edited according to this SOP. Peaks interpreted as non-allelic may be deleted within GMIDX and will appear on the electropherogram with a single strikeout.

5.1.1.1 Identification of Peaks of Non-Genetic Origin

Before the STR typing results from a sample can be used for comparison purposes, it is necessary to identify any non-genetic peaks that do not represent human, allelic STRs. These non-genetic peaks may be undesired PCR products (e.g., stutter, -A, and non-specific product), analytical artifacts (e.g., spikes and raised baseline), instrumental limitations (e.g., matrix failure), or be introduced into the process (e.g., disassociated primer dye and non-specific peaks).³ Additionally, data may have distinctive characteristics consistent with non-human DNA. The various types of peaks are described below.

Reproducible peaks (e.g., stutter, -A, disassociated dye, matrix failure, non-specific product) may be interpreted.⁴ Non-reproducible peaks (e.g., spikes and raised baseline) must be evaluated as specified.

³ The GMIDX software may apply a filter that removes labels from peaks at any locus that meet the FBI-defined sizing and relative peak height criteria for stutter, and 10% for minus-A.

⁴ For purposes of interpreting DNA typing results, a peak need only be identified as being of non-allelic origin.

5.1.1.1.1 Stutter

The kit-specific stutter percentage guidelines provided in Table 3 are estimates of the maximum expected stutter values at each locus in the GlobalFiler™ amplification kit. These values are expressed as a percentage relative to the source allelic peak height (i.e., % Stutter). Stutter may occur at other locations, such as minus-two repeat units, or be excessively high for a sample. If such atypical stutter peaks are due to excessive amounts of template DNA, the sample may be reamplified with less template DNA or reinjected for less time. These values are the stutter percentages incorporated into GMIDX when the stutter filter is used. STRmix™ models stutter using a different method.

Locus	- 1 Repeat Unit	-2 Repeat Units	- 2bp	+1 Repeat Unit
D3S1358	11	1	N/A	2
vWA	11	1	N/A	3
D16S539	10	2	N/A	3
CSF1PO	10	N/A	N/A	6
TPOX	6	N/A	N/A	N/A
D8S1179	10	2	N/A	3
D21S11	11	3	N/A	3
D18S51	13	2	N/A	5
DYS391	8	2	N/A	5
D2S441	8	1	N/A	2
D19S433	10	2	N/A	3
TH01	4	N/A	N/A	N/A
FGA	12	2	N/A	4
D22S1045	18	2	N/A	8
D5S818	10	2	N/A	2
D13S317	10	1	N/A	3
D7S820	9	2	N/A	4
SE33	14	2	5	5
D10S1248	12	2	N/A	4
D1S1656	12	3	3	3
D12S391	14	2	N/A	3
D2S1338	12	2	N/A	4

**Table 3 – Maximum Expected Stutter Percentage Guidelines
for STR Loci Analyzed Using the GlobalFiler™ Kit**

5.1.1.1.2 Minus-A (–A)

It is expected that –A generally occurs at less than 15% for peaks that are not off-scale. The interpretation of –A peaks is based on the following:

- (1) whether the DNA results are derived from a single contributor or whether the N-1 peak may represent an allele from an additional contributor(s) to the sample (this determination will entail multiple loci), (2) the size and relative peak height of the N-1 peak, (3) whether an additional allele(s) at the same locus exhibits an N-1 peak, and (4)

whether there is any indication of excessive DNA template and/or amplification inhibition.

- b. If –A peaks are extensive and/or interfere with interpretation, the sample may be reinjected or reamplified.

5.1.1.1.3 Pull-Up Resulting from Spectral Failure

Pull-up is assessed using the following criteria.

- a. The resultant artifactual peak(s) (usually less than 10% of the source peak) typically sizes within ± 0.25 bp (\pm approximately two scan units) of its source peak. The sizing of a pull-up peak may be distorted if it occurs sufficiently close to another peak in the same color (i.e., the two peaks overlap with respect to the X-axis). Such peaks may display a size difference of greater than ± 0.25 bp relative to its source peak.
- b. Samples exhibiting extensive spectral failure or spectral failure that interferes with interpretation may be reinjected for less time or reamplified with less template DNA.

5.1.1.1.4 Spikes

- a. Spikes are generally detected in two or more colors and typically size within ± 0.15 bp of each other. The morphology of spikes can vary greatly from that of an allelic peak. Depending on whether a spike sizes within a bin it may be labeled as either an allele or an off-ladder (OL) allele.
- b. If a spike occurs at or sufficiently close to an internal size standard peak or a ladder peak such that sizing and/or allelic designation is affected, these samples must be reinjected.
- c. Because a spike(s) within a sample or control (i.e., extraction control and/or negative amplification control) from which no typing results are obtained may mask an allelic peak(s), any such sample or control that displays a spike(s) within the expected size range of a locus that yields relatively small allelic fragments (generally 200 bp in size or less) must be reinjected. Spikes that occur within the size ranges of genetic loci that yield larger allelic fragments may be reinjected.

5.1.1.1.5 Dissociated Primer Dye

The interpretation of dye peaks is based on the following:

- a. The peak's morphology (e.g., dye peaks are generally much broader than peaks of DNA origin).
- b. The presence of peaks of similar size and morphology in other samples and/or controls amplified together with the sample or control being evaluated.

- c. Whether the observed peak occurs within a size range previously associated with a dye peak from a previous lot of amplification kit.

5.1.1.1.6 Non-Specific Peaks

Non-specific peaks are defined as peaks of unknown origin. Some of those known to be associated with specific commercial products are noted below. Additional artifacts are described in the product user guides or technical notes distributed by the manufacturer.⁵

- a. A reproducible artifact intrinsic to the GlobalFiler™ Kit may be detected at the TH01 locus, typically sizing approximately 12 nucleotides smaller than a TH01 allele. The kit manufacturer specifies that these artifacts range from 0.4% to 0.9% of the TH01 allele peak heights.
- b. A reproducible artifact intrinsic to the GlobalFiler™ Kit may be detected at the TPOX locus, typically sizing approximately 24 nucleotides smaller than a TPOX allele.
- c. A reproducible artifact intrinsic to the GlobalFiler™ Kit may be detected between the amelogenin and Y indel loci, typically sizing at ~94.5bp, at 0.3% to 0.7% of the X allele peak and not falling within an allelic bin. The peak height is directly proportional to the peak height of the X allele peak.

5.1.1.1.7 Raised Sample Baseline

A sample(s) that displays excessive raised baseline (i.e., an elevated horizontal axis at, or between, two or more loci that results in stretches of non-specific, low amplitude data) should be reinjected using the standard injection time or for reduced time(s). If such subsequent injections do not result in on-scale data, the sample(s) may be reamplified with less DNA template and/or reextracted.

5.1.1.1.8 Non-Human Peaks

The amplification of non-human DNA may exhibit characteristics such as:

- a. A peak at ~98 bp (before amelogenin) and/or randomly at other loci.
- b. A peak at ~207 bp detected at the D21 locus.
- c. A peak at ~204 bp detected at the vWA locus.
- d. Alleles absent at the majority of the locations but not consistent with a degraded profile or low level human DNA sample.

Non-human DNA typing results should not be used for comparisons.

5.1.1.2 Excessive DNA Template and Off-Scale Samples

An excessive amount of template DNA may result in the appearance of off-scale peak(s), which exceed the linear dynamic range of the genetic analyzer detector. These samples may also exhibit

⁵ See, for example: ThermoFisher Scientific. Technical Note: Artifacts Identified Post-Developmental Validation: Gloabaler™ PCR Amplification Kit. 2019. Available at www.thermofisher.com.

raised baseline, pull-up peaks, -A, atypical stutter, and/or non-specific peaks in one or more colors. If the artifacts are excessive, these samples may be reinjected for reduced time(s), reamplified with less DNA template, and/or reextracted. Off-scale peaks may be displayed by the major contributor to a mixture of DNA when a large difference exists between the major and minor contributions to the sample. In such cases, allelic peaks from the minor contributor may be interpreted in the presence of off-scale major contributor peaks provided that no excessive raised baseline and/or excessive non-specific peaks are present. If necessary, a locus exhibiting excessive non-specific peaks may be deemed inconclusive for matching/statistical purposes.

5.1.1.3 Off-Ladder (OL) Alleles

If an allele fails to size within a defined allele category (e.g., a bin or a virtual bin), it must be assessed using the following criteria.

5.1.1.3.1 Any sample containing an OL allele may be reinjected.

5.1.1.3.2 An OL allele may be a microvariant that sizes between two ladder alleles. For example, if an OL allele occurs between the 12 and 13 ladder alleles and is approximately 1 bp larger than the 12 allele, it is designated as 12.1; 2 bp larger is designated 12.2; and so on.

5.1.1.3.3 If an OL allele does not fall within the size range of any locus-specific ladder, which includes the flanking virtual bins, it must be associated with one of the two loci between which it falls.

- a. For single-source samples, if the OL allele is flanked by a locus with two peaks and a locus with a single peak, the OL allele is assigned to the latter locus.
- b. If an OL allele falls between two loci that both display either a single allele or two or more alleles, the OL allele may be assigned to the locus closest in size to the OL allele. Peak height evaluation may also aid in the assignment of the OL allele to a locus.⁶
- c. If determination of the locus assignment is not possible, both loci that flank the OL allele must be deemed inconclusive for matching/statistical purposes.
- d. If the OL allele is smaller in size than the smallest respective virtual bin, or larger in size than the largest respective virtual bin, the number of repeats in the allele should be estimated for use in STRmix™ analysis.
- e. When loci are closely spaced on the x-axis of an electropherogram, an above or below OL allele may be observed within the size range of a flanking locus. Peak heights, peak height ratios, the number of alleles in the flanking loci, and the size of the OL allele (e.g., whether it is approximately an integer value above or below the flanking ladder allele(s)) may be used to determine the appropriate locus designation for the OL allele.

5.2 Contamination Assessment

Although appropriate quality assurance practices are stringently applied and enforced, it is not unexpected that low-levels of adventitious DNA may be detected due to the highly sensitive nature of the amplification process. Adventitious DNA may be attributable to a specific source (e.g., laboratory

⁶ To facilitate the interpretation of OL alleles, the Examiner may consult a listing of such alleles recorded at http://www.cstl.nist.gov/div831/strbase/var_tab.htm.

personnel) or be from an unknown source. Further, contamination may occur in either a random or systemic manner. Instances of contamination will be evaluated on an individual basis according to the following guidelines.

5.2.1 General Contamination Guidelines

5.2.1.1 If contamination occurs in a sample or control, the affected extraction batch and amplification batch should be assessed for random or systemic contamination. It is noted that systemic contamination may be limited to the extraction batch, leaving the remaining samples in the amplification batch unaffected. The determination of contamination as random or systemic may be complex; the Examiner should consult the Technical Leader (TL) for additional guidance as necessary.

5.2.1.2 If a control is affected by contamination, but the results from the sample indicate that only the expected contributor(s) was present or no results were obtained, the sample does not have to be reprocessed.

5.2.1.3 Other samples should be reprocessed as described:

- If the contamination is characterized as systemic, any sample processed in parallel with the systemic contaminant should be reprocessed if possible.
- If the contamination is characterized as random and occurs in a control, any sample processed in parallel with the affected control should be reprocessed if possible.
- If the contamination is characterized as random and occurs in a sample, the sample should be reprocessed if possible. It is not necessary to reprocess other sample(s) processed in the same batch.

5.2.1.4 If reprocessing is not conducted or possible, the resulting data may be interpreted in a conservative and cautious manner following the procedures described below.

5.2.1.5 Refer to the report wording section 5.5.7 for further direction on how to denote the contamination in the report.

5.2.2 Evidence Handler Contaminant

5.2.2.1 If the contamination event is consistent with having arisen from an individual who handled the sample at any stage of the process, and if reprocessing the sample is not possible or successful, it is possible to condition on the source of the contaminant. Conditioning on the source of the contaminant requires that the typing results support the presence of the contributor in the sample. The results may be used for interpretive purposes and for submission to the Combined DNA Indexing System (CODIS) with TL approval.

5.2.3 Random Contamination

5.2.3.1 If the contamination event is determined to be random, the samples processed in parallel may be used for interpretive purposes and may be submitted for inclusion in CODIS.

5.2.3.2 If a sample with random contamination is the basis for the conclusions in a report (i.e., reextraction was not possible or not successful), the occurrence of contamination must be noted in the report.

5.2.3.2.1 If a single-source or major contributor in an evidentiary sample is determined to be a random contaminant (with the exception of an evidence handler contaminant, see 5.2.2), the results for the single-source or major contributor are not suitable for interpretive purposes and may not be submitted for inclusion in CODIS. Results for a minor contributor(s) may only be used for exclusionary purposes and may not be submitted for inclusion in CODIS.

5.2.3.2.2 If a minor contributor to a mixture in an evidentiary sample is determined to be a random contaminant (with the exception of an evidence handler contaminant, see 5.2.2), the results from that sample for a major contributor may be used only for exclusionary purposes and may not be submitted for inclusion in CODIS.

5.2.4 Systemic Contamination

5.2.4.1 If the contamination event is determined to be systemic (with the exception of an evidence handler contaminant, see 5.2.2), the data for all of the samples processed in parallel may not be used for interpretive purposes and may not be submitted for inclusion in CODIS as this demonstrates a failure in the analytical process. The occurrence of systemic contamination must be noted in the report.

5.2.4.2 With TL approval, exculpatory results (e.g., ability to exclude the subject from the male profile in a sperm fraction) should be reported. The contamination and the limitations of future comparisons must be noted in the report.

5.3 Application of Peak Height Thresholds to Allelic Peaks

5.3.1 The analytical threshold (AT) is 150 RFU; however, it can be lowered to 50 RFU for bones, tissues, hair, and/or teeth, as long as these samples present as single source. When a sample is analyzed with an AT of 50 RFU, the negative amplification control and the reagent blanks associated with the sample must also be analyzed using an AT of 50 RFU. The positive amplification control and ladders will be analyzed using an AT of 150 RFU.

5.3.2 The stochastic threshold (ST) is 725 RFU.

5.3.3 Because STRmix™ incorporates an empirically determined probability of dropout, no ST is used in the interpretation of samples subjected to direct comparisons using STRmix™. However, the ST and potential for allele dropout must be considered during visual comparisons that are the basis for a direct exclusion without the use of STRmix™.

5.3.4 The ST is applicable to typing results from questioned specimens that are used for familial comparisons and known specimens. For samples known or expected to be of single source origin (e.g., reference samples, alternate reference samples, bones, tissue) that display results consistent with having arisen from a single individual, the ST is applied to only those loci at which stochastic loss of information is possible (i.e., loci that display a single allelic peak < ST).

5.4 Interpretation of DNA Typing Results

To the extent possible, DNA typing results from evidentiary samples will be interpreted before the comparison with any known samples, other than those of assumed contributors.

When there are multiple amplifications and/or injections for a given sample extract, generally the one that provides the most information will be used for reporting. However, sample saturation or loss of resolution that interferes with interpretation may require that an alternative amplification/injection is used.

5.4.1 Peak Height Ratio

Peak height ratios (PHR) can be used to associate two alleles to a common source and to establish the presence of a DNA mixture.

5.4.1.1 Peak height ratios are calculated by dividing the peak height of the allele with the lower RFU value by the peak height of the allele with the higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage.

5.4.1.2 The values provided in Table 4 are estimates for the minimum expected PHR percentages for the GlobalFiler™ Amplification Kit and are based on the average PHRs observed in the validation studies. The PHR guidelines are only applicable to allelic peaks that meet or exceed the ST.

Peak Height	All GlobalFiler™ STR Loci
725 – 1999 RFU	50%
2000 – 4999 RFU	60%
5000 RFU and above	70%

Table 4 – Minimum Expected Heterozygous Peak Height Ratio Guidelines

5.4.1.3 Because reference samples and human remains (e.g., bones, teeth, tissue) are attributable to a single individual, PHR assessments are generally not used in their interpretation. Should a mixture of DNA be obtained from such samples, the major contributor type can be assessed using the peak height ratios described in Table 4. A major contributor profile may only be used for an alternate reference sample if no other samples are available. Mixtures in human remains samples will not be submitted to CODIS unless approved by the TL.

5.4.2 Determination of the Number of Contributors to DNA Typing Results

5.4.2.1 The determination of the number of contributors to DNA typing results begins with counting the allelic peaks; therefore, peaks that exceed the expected stutter percentages must be evaluated considering:

- A peak significantly above the stutter percentage is more likely to be allelic.
- A peak at a small (<200 bp) locus where possible minor contributor types are expected has more potential to be allelic.

- A peak in an additive stutter position, which exceeds the negative stutter percentage but not the combined positive and negative stutter percentages, may be considered stutter.
- Other apparent peaks below the AT suggest that the peak is potentially allelic.
- If the sample is a reference sample and expected to be single source, then these peaks can confidently be called stutter if there is no other evidence of contamination.

5.4.2.1.1 For apparent single source samples, a peak in a stutter position that exceeds the expected stutter percentage may be interpreted as a stutter peak for purposes of determining the number of contributors to the sample. Generally, this interpretation is limited to a single instance unless the peaks are in the additive stutter position, at a large (>200 bp) locus, and there are no other indications of a mixture (e.g., peak height imbalance, apparent peaks <AT).

5.4.2.1.2 For mixed samples, peaks that exceed the expected stutter percentage are generally considered allelic for purposes of determining the number of contributors to the sample.

5.4.2.2 Using the locus with the largest number of alleles, divide the number of alleles by two, and round up to the nearest integer. This integer is the initial estimate of the number of contributors to the sample.

5.4.2.3 Again using the locus with the largest number of alleles, assess the ratio of contributors. Evaluate peak height imbalance and account for allele sharing to determine if the number of contributors should be increased. PHR assessments should generally be within empirically determined values for single source samples.⁷

5.4.2.4 Apply the general pattern of number of contributors and mixture ratio across the profile to determine if other loci are consistent with this pattern or if the number of contributors should be increased or decreased by one. Loci with more alleles will be the most informative for this assessment. Additionally, apparent peaks <AT may also be considered, especially for low level samples.

5.4.2.5 Samples in which three allelic peaks⁸ are observed at a single locus, without any other indications of a mixture, may be concluded to be single source.

5.4.2.6 STRmix™ input files will include peaks in the one repeat unit forward or reverse stutter position, while peaks that have been interpreted as stutter at more than one repeat unit or at the -2 bp stutter position will remain filtered by GMIDX.

⁷ Peak height imbalances may be seen in the results from a single individual due to elevated stutter, primer binding site variants that result in attenuated amplification of one allele of a heterozygous pair, or tri-allele patterns in which two copies of an allele are present within the genotype (e.g., a type 11,12,12).

⁸Observed tri-allele patterns are recorded at http://cstl.nist.gov/biotech/strbase/tri_tab.htm.

5.4.3 Sex Determination

Three loci in the GlobalFiler™ kit: Y indel, amelogenin, and DYS391, are used to determine whether male and/or female DNA is detected in a sample. Generally, male DNA will exhibit characteristic alleles at all three loci; however, an allele at only one or two Y-chromosome loci indicates the presence of male DNA. Missing alleles may be due to low quantity, degradation, or primer binding site mutations. The presence of male DNA in a mixed sample may limit the ability to determine if female DNA is also present in the sample.

5.4.3.1 Only female DNA is detected in a single source or mixed sample that exhibits both:

- a) an X peak \geq ST at amelogenin in the absence of a Y peak and
- b) no alleles at Y indel or DYS391

5.4.3.2 No conclusion regarding sex determination can be drawn if the sample exhibits both:

- a) at amelogenin, an X peak $<$ ST in the absence of a Y peak and
- b) no alleles at Y indel or DYS391

5.4.3.3 Male DNA is detected in a sample that exhibits any of the following:

- a) an allele at Y indel
- b) a Y peak at amelogenin (with or without an X peak) and/or
- c) an allele at DYS391

The sample should be reported as single source male, a mixture of male and female, or a mixture containing male DNA based on the following criteria:

5.4.3.3.1 If the sample is single source, it should be reported as male if any male characteristic alleles are detected, regardless of peak height.

5.4.3.3.2 If the sample is mixed, it should be reported as a mixture containing male DNA if either:

- a) both the X and Y peaks are \geq ST and the Y/X PHR is \geq 50% or
- b) both the X and Y peaks are $<$ ST

5.4.3.3.3 If the sample is mixed, it should be reported as a mixture containing male and female DNA if the Y/X PHR is $<$ 50% and either:

- a) the X peak is \geq 1450 (i.e., twice the ST), regardless of the peak height of the Y, or
- b) both the X and Y peaks are \geq ST

5.4.3.3.4 If the sample is mixed, it may be interpreted as a mixture containing male DNA or containing male and female DNA when both:

- a) the X peak is $<$ 1450 (i.e., twice the ST) and
- b) the Y peak is $<$ ST

The FE should consider Y/X PHR, the proportionate heights of the Y indel peak and/or the DYS391 peak, and the profile as a whole to make this determination.

5.4.3.4 For any sample with no alleles at amelogenin, Y indel, and DYS391, no sex typing results were obtained.

5.4.4 Exclusions Based on Visual Comparison of DNA Typing Results

Some exclusions may be declared upon visual comparison of typing results, as described below. No STRmix™ analysis is performed for exclusionary conclusions determined from visual comparisons.

5.4.4.1 Exclusions from Single-Source Profiles

An exclusion is declared when one or more loci in a single-source evidentiary profile is inconsistent with that of a known individual, considering dropout as applicable.

5.4.4.2 Exclusions from Mixed DNA Typing Results

5.4.4.2.1 To declare an exclusion upon visual comparison, the Examiner should consider the number of contributors, the number and height of alleles detected per locus, the height of stutter peaks, the potential for allele sharing among contributors, and the potential for allele dropout. Generally, the greater the complexity of the typing results (e.g., 3 or more contributors, few alleles detected per locus relative to the number of contributors, trace contributor(s), substantial peak imbalance), the greater the potential that the results should be interpreted using STRmix™.

5.4.4.2.2 Based on visual comparison, the Examiner may declare an exclusion to a mixed evidentiary profile if the known profile is not consistent with the potential genotype(s) in the mixture.

5.4.5 DNA Typing Results Subjected to STRmix™ Interpretation

5.4.5.1 STRmix™ analysis may be conducted on evidentiary typing results amplified using the GlobalFiler™ Kit with 28 cycles and separated on the Applied Biosystems 3500xL. Reference profiles are not constrained by these requirements for STRmix™ analysis.

5.4.5.2 STRmix™ is used for interpretation and statistical assessment of typing results from which a POI is not excluded based on visual comparisons. STRmix™ may also be used for mixture deconvolution in the absence of DNA typing results for a known individual.

5.4.5.3 Typing results assumed to originate from 1 to 4 contributors may be interpreted using STRmix™. Mixtures of DNA from 5 or more contributors will not be interpreted using STRmix™, and will be reported as unsuitable for comparisons.

5.4.5.4 A given sample may be interpreted in STRmix™ more than once if alternate hypotheses and assumptions relevant in the context of the case are being assessed.

5.4.5.5 Replicate amplifications of the same extract, with the same or different DNA template quantities, may be interpreted concurrently in the same STRmix™ analysis.

5.4.5.5.1 If a different extract is used for reamplification, the typing results cannot be analyzed as replicates in STRmix™, with the exception of bones, tissue, hair, and teeth with results that present as single source.

5.4.6 STRmix™ Settings

5.4.6.1 STRmix™ software settings, including FBI specific parameters from internal validation studies, are represented in Appendix B and Appendix F.

5.4.6.2 The value for Markov chain Monte Carlo (MCMC) accepts is set to 100,000 for burn-in and 500,000 total, and will not be changed without approval of the TL.

5.4.6.3 An F_{ST} setting of 0.01 is used for the African American, Caucasian, Southeastern Hispanic, Southwestern Hispanic, Chamorro, Filipino, and Trinidadian populations. For Native Americans populations (i.e., Apache, Minnesota Native American, and Navajo), an F_{ST} of 0.03 is used.

5.4.7 Propositions for Calculating the Likelihood Ratio (LR)

5.4.7.1 STRmix™ establishes two propositions (also referred to as hypotheses) based on user input to calculate the LR.

- The first proposition, H_1 , generally includes the POI and, for mixed specimens, unrelated unknown (U) individuals. The total count of individuals included in the proposition is equal to the number of contributors in the sample.

Example: For a three-person mixture, H_1 consists of the POI and two unrelated unknown individuals.

- The second proposition, H_2 , generally consists of unrelated unknown individuals, equaling in total the number of contributors to the sample.

Example: For a three-person mixture, H_2 consists of three unrelated unknown individuals.

5.4.7.2 Conditional propositions are used for mixed typing results when the presence of an individual's DNA in the sample can be reasonably expected, and the typing results support the presence of the contributor in the sample. In an analysis conditioned on a known individual, H_1 and H_2 include the same assumed contributor.

Example: for a three-person mixture:
 H_1 = Assumed contributor + POI + U
 H_2 = Assumed contributor + U + U

The assumed contributor(s) must be the first reference sample added to STRmix™. The assumed contributor(s) must be designated in H_2 using the “Change Hd” function in STRmix™. When an assumed contributor(s) is designated in H_2 , STRmix™ reduces the number of unrelated unknown individuals as appropriate.

5.4.7.3 For cases with multiple POIs, each POI reference profile is analyzed individually in STRmix™ and reported separately. A request or scenario where it may be informative to report the LR of combined POIs must be approved by the TL.

Example: for a two-person mixture with POI₁ and POI₂:

$$\begin{array}{ll} H_1 = \text{POI}_1 + U & \text{and} \quad H_1 = \text{POI}_2 + U \\ H_2 = U + U & H_2 = U + U \end{array}$$

5.4.7.3.1 If multiple POIs yield inclusionary LRs (>1), the results should be assessed to ensure that the included POIs could be present in the mixture together. This assessment will be done either visually (e.g., two POIs together do not account for all alleles in a two person mixture) or by running STRmix™.

5.4.7.3.2 If included POIs are interpreted to fit together in the mixture, they must be analyzed in STRmix™ together in a single analysis.

Example: for a three-person mixture with inclusionary POI₁ and POI₂:

$$\begin{array}{l} H_1 = \text{POI}_1 + \text{POI}_2 + U \\ H_2 = U + U + U \end{array}$$

5.4.7.3.3 If STRmix™ analysis of the combined POIs indicates that they can be present together in the mixture, the LRs run individually should be reported for each POI, and the combined LR should be maintained in the casefile.

5.4.7.3.4 If visual comparison (e.g., a limited single source profile that provides inclusionary LRs for 2 POIs) or STRmix™ analysis of the combined POIs indicates that they cannot be present together under the assumptions of the analysis, the profile and diagnostics may be reassessed to determine whether the analysis should be repeated under the same or different conditions (e.g., increasing the assumed number of contributors). If no change to the analysis is supported by the profile or diagnostics, the report should indicate this circumstance directly following the H_1 supporting LR table. For example:

Under the assumption that the DNA from item 1 originated from two individuals, it is not possible for both WHITE and JONES to be contributors.

5.4.7.4 Evidentiary samples will be compared to all informative POIs. If there is no expectation that DNA from a particular POI would be present on an evidentiary sample, then no comparison to that POI is required for that sample.

5.4.8 STRmix™ Analysis

5.4.8.1 Information on software usage can be found in the STRmix™ User's Manual.

5.4.8.2 Tri-allelic loci in an evidentiary sample should be excluded from STRmix™ analysis by using the "Ignore locus" function. In a reference profile, alleles in tri-allelic loci should be deleted from the profile prior to import into STRmix™.

5.4.8.3 The Examiner will evaluate the STRmix™ results, including the diagnostics and weights of various genotypes relative to the DNA profile analyzed. In general, each STRmix™ analysis, consisting of the same evidence and reference profiles, propositions, and assumptions, will be run once and the results reported, except when an analysis has produced a result that requires further investigation and reanalysis. Examples of when repeating the STRmix™ analysis is appropriate include:

- An LR = 0 is obtained for a single locus, with other loci having an LR > 0, when the reference profile is consistent with the evidentiary profile. Review of the genotype probabilities may indicate that STRmix™ did not consider all potential genotype sets. Repeat analysis should be conducted with the same STRmix™ settings.
- An LR = 0 is obtained for a single locus, with other loci having an LR > 0, when the reference profile is consistent with the evidentiary profile. Review of the electropherogram indicates that an allele was not included in the STRmix™ analysis (e.g., a 9.3 allele that was not sufficiently resolved from a 10 allele). In this instance, the analysis should be repeated with the locus ignored in STRmix™.
- An observation that does not appear intuitively correct, such as (a) mixture proportions that do not reflect what is observed in the typing results, (b) degradation that does not reflect what is observed, or (c) the interpreted contributor genotypes do not appear intuitively correct.
- The number of contributors to a sample is ambiguous, or the STRmix™ results are not intuitively correct under the assumption of the tested number of contributors. Also, the number could be underestimated if the contributors are related and share alleles. The Examiner may repeat the STRmix™ analysis assuming a different number of contributors.

Should the diagnostics or results indicate that further scrutiny is required, a number of possible rework options are available. For example:

- Repeat STRmix™ analysis as appropriate. Should the original STRmix™ results be caused by a possible software limitation and subsequent results are acceptable, the initial STRmix™ results need not be included in the case file.
- Reassessment of the assumed number of contributors.
- Rerun or reamplification with the same or different amount of DNA template to strengthen the number of contributors assumption or assist with sub optimal PCR performance and/or allele designation.

5.4.8.3.1 If the STRmix™ analysis has been carried out more than once based on alternate propositions and/or assumptions, all analyses will be included in the case file, but the result of the most appropriate analysis in the context of the case⁹ will be reported.

5.4.8.4 If a profile has been analyzed in STRmix™ with acceptable results and a subsequent analysis is performed, such as a comparison to a new reference profile, the subsequent analysis should be performed using the “LR from previous analysis” function in STRmix™.

⁹ Case information available at the time of reporting is used to determine the most appropriate hypotheses and assumptions to include in the report.

5.4.9 Likelihood Ratio (LR)

A variety of LRs are provided in the STRmix™ results output, including those for relatives.

5.4.9.1 Generally, the unrelated highest posterior density (HPD) LR (with the factor of N! enabled) will be reported. However, if analysis in consideration of a relative of a POI is appropriate, the value for the relationship of interest may be reported with TL approval.

5.4.9.2 The settings for LR calculation are shown in Appendix B and Appendix F, to include the Factor of N! LR and the HPD with a one-sided quantile of 0.99.

5.4.9.3 LRs are calculated using four general United States population groups (African American, Caucasian, Southeastern Hispanic, and Southwestern Hispanic). Additional LRs will be calculated for specimens that potentially originate from Native American populations (i.e., Apache, Minnesota Native American, and Navajo), Caribbean populations (i.e., Trinidadian), or Chamorro/Filipino populations.¹⁰

5.4.9.3.1 The allele frequency distributions for the African American, Caucasian, Southeastern Hispanic, Southwestern Hispanic, Apache, Navajo, Trinidadian, Chamorro, and Filipino populations are published in *Forensic Science International: Genetics*.¹¹ The allele frequency distributions for the Minnesota Native American population are included in Appendix D. The African American population includes samples from the African American, Bahamian, and Jamaican populations.

5.4.9.3.2 At any locus, the published allele frequencies are incorporated into STRmix™ for each allele that contains a value. Refer to the STRmix™ User's Manual for information regarding alleles for which no frequency value is available.

5.4.9.4 The single lowest LR (HPD) obtained across all populations used in the calculation will be reported.

5.4.10 Determination of Typing Results Suitable for CODIS¹²

5.4.10.1 Forensic Unknown and Forensic Partial profiles may be entered into CODIS directly. Samples uploaded to the Forensic Mixture specimen category will be deconvoluted using STRmix™ to determine the CODIS profile. Generally, a particular STRmix™ contributor may be entered into CODIS if, at each given locus, the genotype(s) exhibit a (combined) weight of at least 99%. For example, if the STRmix™ results for Contributor 1 indicate the following genotypes and weights for a locus:

¹⁰ The use of Native American, Caribbean, or Chamorro/Filipino population databases is generally based on the geographic location of the requesting agency. The listed Native American or Caribbean population databases are appropriate for use regardless of the specific Native American or Caribbean population group in the case scenario. Statistics for cases originating from Puerto Rico will be calculated using the four general United States population databases and do not require the use of the Caribbean population databases. The Chamorro/Filipino population databases would be used generally for cases originating from the U.S. territories of Guam and the Commonwealth of Northern Mariana Islands (e.g., Saipan).

¹¹ Moretti TR, Moreno LI, Smerick JB, Pignone ML, Hizon R, Buckleton JS, Bright J-A, Onorato AJ. Population data on the expanded CODIS core STR loci for eleven populations of significance for forensic DNA analyses in the United States. *Forensic Science International: Genetics* (2016) 25: 175-181.

¹² DNA typing results may be entered into other appropriate databases.

Contributor 1:

<u>Genotype</u>	<u>Weighting</u>
8, 8	41.31
8, 10	30.11
10, 10	24.51
7, 10	3.92
6, 8	0.15

The total weight of the first four genotypes is 99.85%. Therefore, “7, 8, 10” would be entered into CODIS for this locus. The 6 allele would not be entered. Alternatively, the genotypes for a particular STRmix™ contributor may be taken directly from the “SUMMARY>=99%” table that is a part of the STRmix™ output.¹³

5.4.10.2 In the event that the match rarity for a contributor does not meet the criteria for searching at a particular level for CODIS, the combined weight of the genotypes may be lowered to 95%. In the above example, at 95%, the “8, 10” would be entered into CODIS for this locus. The 6 and 7 alleles would not be entered.

5.5 Suggested Reporting Language

The results and/or conclusions for specimens subjected to DNA analysis will generally be reported in narrative form. The formatting and administrative information required in a report are described in the appropriate *FBI Laboratory Operations Manual* practices and *DNA Procedures Manual* procedures (i.e., DNA 610). For additional guidance on reporting language for Y-STRs or familial comparisons refer to the appropriate interpretation protocols of the *DNA Procedures Manual*.

5.5.1 Introductory Statements

5.5.1.1 Items Analyzed and Amplification Kit(s) Used

The report must indicate (a) that the items were subjected to DNA typing, (b) that PCR methodology was used in DNA analysis, (c) specify which Amplification Kit was used, and (d) that the STRmix™ software was used, if applicable. The report should contain the item listing followed by the general introductory statement:

*“The items listed above were subjected to serological testing and/or nuclear deoxyribonucleic acid (DNA) analysis. * Probabilistic genotyping was performed using the STRmix™ software.”*

With the appropriate associated endnote:

“ DNA typing using the polymerase chain reaction (PCR) of short tandem repeats (STRs) was performed with the GlobalFiler™ PCR Amplification Kit.”*

¹³ STRmix™ rounds weightings to determine whether a genotype is included in this table; it is possible that an included genotype will be just less than 99%. However, these genotypes are sufficient for CODIS purposes.

“ DNA analysis using the polymerase chain reaction (PCR) was performed with the Quantifiler™ Trio DNA Quantification Kit.”*

If the DNA typing results will be included in the report, they should generally be included as follows:

“The DNA typing results are detailed below:”

Locus	JONES
D3S1358	15, 16
vWA	14, 16
D16S539	9, 10
CSF1PO	11, 12
TPOX	8
Y indel	2
Amelogenin	X, Y
D8S1179	12, 13
D21S11	28, 31
D18S51	12, 15
DYS391	11
D2S441	14, 15
D19S433	14, 15
TH01	7, 9.3
FGA	24, 26
D22S1045	11, 16
D5S818	11
D13S317	11
D7S820	7, 12
SE33	17, 25.2
D10S1248	12, 15
D1S1656	13, 16
D12S391	18, 19
D2S1338	20, 23

5.5.1.2 Comparisons to Previously Reported Results

Generally, one of the following statements should be included under the results section of the report to identify item(s) previously subjected to DNA typing but for which the results of additional comparisons are being reported:

“The DNA typing results from items 1 and 2 were compared to the DNA typing results from SMITH [previously reported under FBI Laboratory Number 2020-01234-2 in the report dated July 26, 2020]. The results for JONES were also included in the report dated July 26, 2020.”

“The DNA typing results from SMITH were compared to the DNA typing results from item 1 [previously reported under FBI Laboratory Number 2020-01234-1, FBI Case Identification number 95A-HQ-0123456, in the report dated July 26, 2020].”

“Items 1, 2, and 7 (JONES) were previously reported under FBI Laboratory Report 2020-01234-2 on July 26, 2020. The DNA typing results from SMITH were compared to the DNA typing results from items 1 and 2. The conclusions reported for items 1 and 2, in the report dated July 26, 2020, are being amended as a result of implementing new interpretation guidelines that utilize probabilistic genotyping software.”

“The DNA typing results from the items listed above were compared to the DNA typing results from SMITH [previously reported under FBI Laboratory Number 2020-01234-2 in the report dated July 26, 2020].”

“The DNA typing results from items 1 and 2 were compared to the DNA typing results from SMITH [provided by the New Jersey State Police Office of Forensic Sciences on December 16, 2019].”

5.5.1.3 Alternate Reference Samples

The use of a questioned item of established origin as an alternate reference sample should generally be stipulated in the report as follows:

“It is noted that for comparison purposes, item 1 is being used as an alternate reference sample for JOHN SMITH.”

“Per incoming communication dated November 20, 2020 from Special Agent Jane Doe, item 7 (shirt) is being used as an alternate reference sample for JOHN SMITH.”

“Per communication with Special Agent Jane Doe on November 20, 2020, item 7 (shirt) is being used as an alternate reference sample for JOHN SMITH.”

“Assuming the major contributor to item 1 is JOHN SMITH, the DNA profile of the major contributor to item 1 is being used as an alternate reference sample for JOHN SMITH.”

5.5.1.4 Elimination Samples

The use of a reference item identified by the contributor as an elimination sample, such as a consensual partner or an evidence technician, should generally be captured in the report as follows:

“Per incoming communication dated May 15, 2020 from SA Michael Jones, BROWN was provided as an elimination sample for comparison to items 3 through 10.”

The elimination sample should be used for comparison and reporting if:

- There is no DNA unlike the elimination sample.
- The elimination sample is included and is conditioned upon for STRmix™ purposes.
- The elimination sample is visually excluded.

If based on the results, the elimination sample is not used for conditioning and no visual exclusion is possible, additional language should be added:

However, based on the nature of the results, BROWN was not required for the interpretation of items 4 and 8.

This additional language is not needed if no results are obtained for an item.

5.5.1.5 Relationships

For missing person reports, a statement should generally be included to define the relationships of the submitted reference samples to the missing person as follows:

“It is noted that SARAH SMITH is identified by the incoming communication from the contributor as the biological mother of the missing person JANE SMITH.”

5.5.1.6 Differentially Extracted Samples

5.5.1.6.1 The designations 1F and 1M may be truncated to the original item identifier (e.g., item 1) for reporting purposes. Differentially extracted samples that result in distinct fractions may be reported together where the fractions are treated as individual contributors to a mixture. This approach may also be used if the DNA typing results from both fractions are the same. DNA types consistent with carry-over from either fraction may be subtracted from the opposite fraction by using the profile as a conditional reference sample in STRmix™.

5.5.1.6.2 The designations 1F and 1M may be maintained if it is necessary to report the DNA typing results from each fraction separately. An explanatory statement should be included in the report as follows:

“Item 1 was extracted in two fractions, which will be designated items 1M and 1F.”

5.5.2 Reporting STRmix™ Results and Conclusions

5.5.2.1 Each DNA association must be clearly and properly qualified with either a statistic or a qualitative statement. STRmix™ analysis is performed to provide a statistic. A qualitative statement not based on a statistical calculation should be limited to situations in which the presence of an individual's DNA on an item is reasonably expected. The provenance of the sample must be established in the case record when statistics are not calculated.

5.5.2.2 The sex typing results for a sample should be reported based on the three sex determining loci: amelogenin, Y indel and DYS391 (see 5.4.3).

5.5.2.3 For a given STRmix™ analysis, the assumption as to the number of contributors (N) and any individuals assumed to be present in the sample (conditioned contributors) should be reported. The assumed number of contributors in the report should match the STRmix™ analysis.

5.5.2.4 The single lowest HPD LR value across all appropriate populations is used for drawing conclusions, and it is generally truncated to two significant digits for reporting. However, if this value is between 1/10 and 10, it is truncated to one significant digit for reporting. For $1/100 < LR < 1$ (i.e., $0.01 < LR < 1$), the reciprocal should be calculated prior to truncating for reporting.

5.5.2.5 The magnitude of the LR relates to the degree of support provided by the evidence under the tested hypotheses and assumptions. A qualitative statement should be reported based on the following table:

LR	Qualitative Equivalent
0 to 1/100	Exclusion
>1/100 to 1/2	Limited support for Exclusion
1	Uninformative
2 to <100	Limited support for Inclusion
100 to <10,000	Moderate support for Inclusion
10,000 to <1,000,000	Strong support for Inclusion
$\geq 1,000,000$	Very strong support for Inclusion

5.5.2.6 LRs $>1/100$ (i.e., 0.01) will have a statement included in the report and should also be reported in tabular form, except when $LR=1$. The table should include the name of the POI(s), any assumed contributors, the LR, and the qualitative level of support for the conclusion.

5.5.2.7 All exclusions, visual exclusions and $LR \leq 1/100$ (i.e., 0.01), may be reported using a bulleted list of the excluded POI(s). The LR value need not be reported for exclusions.

5.5.2.8 Report Wording Examples

Results and conclusions are generally preceded by a description of the item tested and/or, where appropriate, the sampling area tested from an item.

See section 5.5.2.9 for endnote language, denoted A-E in these examples.

Item 1 (Pants from Jones)

Item 1(1) (Bloodstain from pants)

Male DNA is present in item 1(1). Item 1(1) was interpreted as originating from one individual.

The DNA results from item 1(1) are 74 sextillion times more likely if JAMES is a contributor than if an unknown, unrelated person is a contributor.

Person of Interest (POI)	Likelihood Ratio (LR) ^A	Level of Support ^B
JAMES	7.4×10^{22} (74 sextillion)	Very strong support for Inclusion

The following individuals are excluded as potential contributors to item 1(1):^C

- GARCIA
- JONES
- WHITE

Item 1(3) (Stain from pants) [support for H_2 changes the sentence before the table]

No conclusion regarding sex typing results can be provided for item 1(3). Item 1(3) was interpreted as originating from two individuals.

The DNA results from item 1(3) are 40 times more likely if two unknown, unrelated people are contributors than if JAMES and an unknown, unrelated person are contributors.

Person of Interest (POI)	1/Likelihood Ratio (1/LR) ^A	Level of Support ^B
JAMES	40	Limited support for Exclusion

The following individuals are excluded as potential contributors to item 1(3):^C

- GARCIA
- JONES
- WHITE

Item 1(5) (Stain from pants) [no table for LR=1]

Male DNA^D is present in item 1(5). Item 1(5) was interpreted as originating from two individuals.

The DNA results from item 1(5) are equally likely if JONES and an unknown, unrelated person are contributors than if two unknown, unrelated people are contributors.^E

The following individuals are excluded as potential contributors to item 1(5):^C

- GARCIA
- JAMES
- WHITE

Item 3 (Swab of wall) [if all have same (H_1 or H_2) support, enter both in the table together and use placeholders; also LR=1 for >1 POI]

Male and female DNA was obtained from item 3. Item 3 was interpreted as originating from three individuals.

The DNA results from item 3 are [LR] times more likely if [POI] and two unknown, unrelated people are contributors than if three unknown, unrelated people are contributors.

Person of Interest (POI)	Likelihood Ratio (LR) ^A	Level of Support ^B
JAMES	2.6×10^4 (26,000)	Strong support for Inclusion
GARCIA	3.4×10^7 (34 million)	Very strong support for Inclusion

The DNA results from item 3 are equally likely if any of the following individuals and two unknown, unrelated people are contributors than if three unknown, unrelated people are contributors.^E

- JONES
- WHITE

Item 3-1 (Swab of wall) [>1 POI in table, all H_2 support; the sentence changes]

Male and female DNA was obtained from item 3-1. Item 3-1 was interpreted as originating from three individuals.

The DNA results from item 3-1 are [1/LR] times more likely if three unknown, unrelated people are contributors than if [POI] and two unknown, unrelated people are contributors.

Person of Interest (POI)	1/Likelihood Ratio (1/LR) ^A	Level of Support ^B
JAMES	83	Limited support for Exclusion
GARCIA	22	Limited support for Exclusion

The following individuals are excluded as potential contributors to item 3-1:^C

- JONES
- WHITE

Item 3-2 (Swab of wall) [different support gets separate table; if >1 POI in a table, then use placeholders in the sentence before]

Male and female DNA was obtained from item 3-2. Item 3-2 was interpreted as originating from three individuals.

The DNA results from item 3-2 are 26,000 times more likely if JAMES and two unknown, unrelated people are contributors than if three unknown, unrelated people are contributors.

Person of Interest (POI)	Likelihood Ratio (LR) ^A	Level of Support ^B
JAMES	2.6×10^4 (26,000)	Strong support for Inclusion

The DNA results from item 3-2 are 75 times more likely if three unknown, unrelated people are contributors than if GARCIA and two unknown, unrelated people are contributors.

Person of Interest (POI)	1/Likelihood Ratio (1/LR) ^A	Level of Support ^B
GARCIA	75	Limited support for Exclusion

The DNA results from item 3-2 are equally likely if any of the following individuals and two unknown, unrelated people are contributors than if three unknown, unrelated people are contributors.^E

- JONES
- WHITE

Item 4 (neck swab) [e.g., 2 person mix assuming White; same if differential extraction and assuming in Male (M) fraction; H_2 support]

Male and female DNA was obtained from item 4. Item 4 was interpreted as originating from two individuals, one of whom is WHITE.

The DNA results from item 4 are 45 times more likely if WHITE and an unknown, unrelated person are contributors than if WHITE and GARCIA are contributors.

Person of Interest (POI)	Assumed Contributor	1/Likelihood Ratio (1/LR) ^A	Level of Support ^B
GARCIA	WHITE	45	Limited support for Exclusion

The DNA results from item 4 are equally likely if WHITE and JONES are contributors than if WHITE and an unknown, unrelated person are contributors.^E

JAMES is excluded as a potential contributor to item 4.^C

Item 5 (vaginal swab) [e.g., clean male in M fraction; clean or mixed female in Female (F) fraction; STRmix™ on the single source clean male]

Male and female DNA was obtained from item 5. Item 5 was interpreted as originating from two individuals, one of whom is WHITE.

The DNA results from item 5 are 8.6 billion times more likely if GARCIA is a contributor than if an unknown, unrelated person is a contributor.

Person of Interest (POI)	Likelihood Ratio (LR) ^A	Level of Support ^B
GARCIA	8.6 x 10 ⁹ (8.6 billion)	Very strong support for Inclusion

The following individuals are excluded as potential contributors to item 5:^C

- JAMES
- JONES

Item 6 (rectal swab) [e.g., clean F fraction, 2 person mix in M, no indication of a 3rd person, but NOT assuming White in STRmix™ – limited to intimate samples (e.g., fingernail samples, neck swabs)]

Male and female DNA was obtained from item 6. Item 6 was interpreted as originating from two individuals, one of whom is WHITE.

The DNA results from item 6 are 8.6 billion times more likely if GARCIA and an unknown, unrelated individual are contributors than if two unknown, unrelated people are contributors.

Person of Interest (POI)	Likelihood Ratio (LR) ^A	Level of Support ^B
GARCIA	8.6 x 10 ⁹ (8.6 billion)	Very strong support for Inclusion

The following individuals are excluded as potential contributors to item 6:^C

- JONES
- JAMES

Item 7 (cervical swab) [e.g., clean F fraction matches White; 2 person mix in M fraction, but 2nd person is not White; 3 people overall (only difference between 6 and 7 is second sentence)]

Male and female DNA was obtained from item 7. Item 7 was interpreted as originating from three individuals, one of whom is WHITE.

The DNA results from item 7 are 37 billion times more likely if GARCIA and an unknown, unrelated individual are contributors than if two unknown, unrelated people are contributors.

Person of Interest (POI)	Likelihood Ratio (LR) ^A	Level of Support ^B
GARCIA	3.7 x 10 ¹⁰ (37 billion)	Very strong support for Inclusion

The following individuals are excluded as potential contributors to item 7:^C

- JONES
- JAMES

Item 10 (Blanket) [e.g., clean M fraction matches Garcia; F fraction is a 2 person mix of M fraction profile and White; reporting separate fractions]

Item 10(1) (Semen stain from blanket)

Item 10(1) was extracted in two fractions, which will be designated items 10(1)M and 10(1)F.

Male DNA was obtained from item 10(1)M. Item 10(1)M was interpreted as originating from one individual.

The DNA results from item 10(1)M are 21 quintillion times more likely if GARCIA is a contributor than if an unknown, unrelated person is a contributor.

Person of Interest (POI)	Likelihood Ratio (LR) ^A	Level of Support ^B
GARCIA	2.1×10^{18} (2.1 quintillion)	Very strong support for Inclusion

Male DNA^D was obtained from item 10(1)F. Item 10(1)F was interpreted as originating from two individuals, one of whom is the male obtained from item 10(1)M.

The DNA results from item 10(1)F are 450 quintillion times more likely if the male from 10(1)M and WHITE are contributors than if the male from 10(1)M and an unknown, unrelated person are contributors.

Person of Interest (POI)	Assumed contributor	Likelihood Ratio (LR) ^A	Level of Support ^B
WHITE	Male from 10(1)M	4.5×10^{20} (450 quintillion)	Very strong support for Inclusion

The following individuals are excluded as potential contributors to item 10(1):^C

- JONES
- JAMES

5.5.2.9 Associated Endnotes for Reporting Language

The following endnotes should generally be used for reporting.

^A The likelihood ratio is a statistical approach that compares the probabilities of observing the DNA results under two alternative propositions. Calculations were performed using the African American, Caucasian, Southeastern Hispanic, and Southwestern Hispanic populations. The lowest calculated likelihood ratio is reported.

^B These likelihood ratio ranges provide the following support for the conclusion:

<u>Likelihood Ratios:</u>	<u>Qualitative Equivalent:</u>
$\leq 1/100$	Exclusion
$>1/100$ to $1/2$	Limited support for Exclusion
1	Uninformative
2 to <100	Limited support for Inclusion
100 to $<10,000$	Moderate support for Inclusion
10,000 to $<1,000,000$	Strong support for Inclusion
$\geq 1,000,000$	Very strong support for Inclusion

^C A person of interest is excluded either visually or when the likelihood ratio is less than or equal to $1/100$. An exclusion means that the person of interest was not detected in the DNA results.

^D The presence of male DNA in a mixture may limit the ability to determine if female DNA is also present in that mixture.

^E This conclusion is drawn when the likelihood ratio is equal to 1; this comparison is uninformative.

5.5.2.10 Terrorist Explosive Device Analytical Center (TEDAC) Report Wording Example

The following example is the formatting and report wording used when reporting results obtained from TEDAC specimens subjected to DNA analysis. These results are generally reported in tabular form, with the associated endnotes F and G, as follows.

Item(s)	Profile suitable for comparison purposes ^F	Database Entry ^G
Item 1	YES	YES
Item 2	NO	
Item 3	NR	

^F The results listed in the table indicate if a DNA profile was generated that is suitable for comparison purposes. NR means that no STR results were obtained from that sample. NO means that a mixture of five or more individuals was obtained and the results are not suitable for comparisons. YES means that the results obtained from that sample are suitable for comparison purposes.

^G The eligible DNA results for the samples marked YES will be entered into the Combined DNA Index System (CODIS) and/or any other appropriate database. When the same or similar DNA typing results are obtained from multiple items, only the item that yields the most complete profile is entered. These results will be maintained by the FBI Laboratory for possible future comparisons.

5.5.3 Unsuitable Results

Mixtures of DNA from 5 or more contributors, will be reported as unsuitable for analysis. Any comparison declared unsuitable for analysis should generally be reported as follows:

“A mixture of five or more individuals was obtained from item 1; therefore, the results are not suitable for comparisons.”

5.5.4 No DNA Results

For samples for which no DNA typing results were obtained, this information should be reported as follows:

“No DNA typing results were obtained from item 1; therefore, no comparisons can be made.”*

“Male (Female) DNA was obtained from item 1. No additional DNA typing results were obtained from item 1; therefore, no comparisons can be made.”*

“No DNA was detected from item 1; therefore, no DNA typing was conducted.”*

with the following endnote:

*“*Insufficient DNA quality and/or quantity can affect the ability to generate a result and is not an absolute determination that an individual did not come into contact with an item of evidence.”*

5.5.5 No DNA Results other than an expected contributor

For samples for which no DNA typing results other than an expected contributor were obtained, this information should be reported as follows:

Female DNA is present in item 1. No DNA typing results unlike SMITH were obtained from item 1.

Male DNA is present in item 5. No DNA typing results unlike JONES were obtained from item 5. Therefore, no comparisons were made to GREEN.

5.5.6 Unknown Subject (UNSUB) Results

If reference samples are not submitted, or if the evidentiary profile does not match any reference samples, the results may be reported as follows along with the appropriate exclusionary statement:

“Item 1 is consistent with originating from a single male (female) individual and is suitable for comparison purposes.”

“The mixture of DNA obtained from item 3 contains female DNA and is suitable for comparison purposes.”

“The mixture of DNA obtained from item 4 contains male DNA and is suitable for comparison purposes.”*

** The presence of male DNA in a mixture may limit the ability to determine if female DNA is also present in that mixture.”*

“The mixture of DNA obtained from item 5 contains male and female DNA and is suitable for comparison purposes.”

“Male and female (Male; Female) DNA was obtained from item 6. The DNA unlike JONES obtained from item 6 is consistent with originating from a single male (female) individual and is suitable for comparison purposes.”

“No conclusion regarding sex typing results can be provided for item 7. Item 7 is consistent with originating from a single individual (mixture of individuals) and is suitable for comparison purposes.”

5.5.7 Contamination

The following wording can be used as a guide to report contamination in the event the samples are not or cannot be reprocessed.

Random contamination:

"The negative control that was processed together with item 1 displayed possible contamination. No sample remains from item 1 for retesting; however, because the DNA results from the possible contaminant are not present in item 1, and therefore not systemic in nature, the DNA results for item 1 were used for comparison purposes."

"DNA consistent with laboratory staff was present in the DNA obtained from item 1. This individual was involved in the processing or handling of item 1, and retesting was not possible. Therefore, this individual was treated as an assumed contributor to the DNA obtained from item 1 and will further be referred to as STAFF."

Systemic contamination:

"The negative control that was processed together with item 1 displayed possible contamination. The DNA typing results obtained from the negative control were observed in one or more samples or controls and therefore, may be systemic."

The systemic contamination statement may be supplemented with the wording below depending upon the specific situation:

"No sample remains from item 1 for retesting; therefore, no conclusion can be offered in regard to item 1."

"Item 1 was not reprocessed per communication with SA James Madison on DATE; therefore, no conclusion can be offered in regard to item 1."

"No sample remains from item 1 for retesting; however, no DNA foreign to SMITH was obtained from item 1."

"No sample remains from item 1 for retesting; however, no DNA was obtained from item 1."

5.5.8 No other examinations

A statement that no additional serology or nuclear DNA examinations were conducted should be made.

5.5.9 CODIS Statements

A statement must be included in the report of DNA examinations that indicates when an item's typing results will be initially entered into the CODIS or other appropriate database. Alternatively, a statement is typically added when no results are entered into CODIS. This information should generally be

reported as follows; however, TEDAC reports may address database entry in the tabular report format (see 5.5.2.10).

CODIS and/or other database entry:

“The eligible DNA typing results for item 1 will be entered into the Combined DNA Index system (CODIS).”

“The DNA typing results for item 1 will be entered into the (Unidentified Human Remains/Missing Persons/Relatives of Missing Persons) Index of the Combined DNA Index system (CODIS).”

“The DNA typing results obtained from the tested items are not eligible for entry into the Combined DNA Index System (CODIS); however, the results from SMITH will be maintained and searched in the appropriate databases.”

No CODIS entry:

“The DNA results obtained from the tested items are not eligible for entry into the Combined DNA Index System (CODIS).”

“Because no DNA typing results were obtained, the tested items are not eligible for entry into the Combined DNA Index System (CODIS).”

5.5.10 Remarks Statements

5.5.10.1 A statement regarding the maintenance of results should be included. For example:

“These results will be maintained by the FBI Laboratory for possible future comparisons.”

5.5.10.2 Request for Reference Samples:

If reference samples are requested, a note may be added:

“If future comparisons are requested, a known blood or buccal (saliva) sample from the victim/subject should be submitted.”

5.5.10.3 Discontinued

At the request of a contributor, examinations may be discontinued after they have been initiated, up to the point that they are loaded onto the 3500xL. If examinations are discontinued, items should be reported, with the reason for the discontinuation included (in this example, a plea agreement), as follows:

“Examinations were initiated on items 1 and 2 and were discontinued on the submitted item(s) per communication with Special Agent Smith on January 20, 2020, due to a plea agreement.”

5.5.10.4 Additional STRmix™ Analysis

If an additional STRmix™ analysis was conducted and those results are maintained in the case file, a note should be added:

“Alternative hypotheses were considered during the STRmix™ analysis of items 1 and 2. These results are included in the FBI file.”

5.5.10.5 Items Not Examined

If items were included in the evidence listing but not examined, the following note should be added:

“Items 3, 5, and 8 were not examined.”

5.5.10.6 Consumption of Evidence

If items were consumed in the course of examinations, the following note should be added:

“Items 1, 2, and 4 were consumed during the DNA Casework Unit examinations.”

5.5.10.7 Evidence Pending Examination

If additional items are pending examination and will be the subject of another report, the following note should be added:

“Additional evidence is pending serological and/or DNA examinations and will be the subject of a separate report.”

6 Limitations

6.1 It is not possible to anticipate the nature of all potential DNA typing results or the nature of the evidentiary specimens from which they may be obtained. These procedures do not exhaust the possible list of the results that may be encountered by the Examiner, nor the conclusions that an Examiner may render based on his/her interpretation of those results. For those results not specifically described, conclusions should be drawn using the procedures given for the results above that are similar in concept and/or origin.

6.2 All reasonable attempts are made by DNA personnel to preserve material (i.e., evidence material and/or isolated DNA) for potential future DNA testing. However, it is sometimes necessary to consume a sample (e.g., a small bloodstain) in its entirety (i.e., no original material or isolated DNA would remain) to ensure that the best attempt possible is made to obtain DNA typing results for comparison purposes. Should the total consumption of a sample be anticipated by an Examiner, or should the performance of any additional examination(s) result in the total consumption of a sample, an Examiner should ensure that the contributing agency or other responsible office (e.g., Office of the

United States Attorney) is contacted concerning this necessity. Based on this discussion, a mutually acceptable strategy should be developed concerning the future testing of such a sample(s).

6.3 In DNA mixtures of closely related individuals (e.g., parents, offspring, and siblings), false inclusions of other closely related family members may occur due to the elevated sharing of genetic information between relatives.

6.4 Mixtures of DNA from 5 or more contributors will not be interpreted using STRmix™, and will be reported as unsuitable for comparisons.

6.5 Loci exhibiting a tri-allelic pattern cannot be assessed using the STRmix™ software. These loci will be ignored during STRmix™ analysis using the “ignore locus” function.

6.6 The presence of male DNA in a mixture may limit the ability to determine if female DNA is also present in that mixture.

6.7 Insufficient DNA quality and/or quantity can affect the ability to generate a DNA typing result and is not an absolute determination that an individual did not come into contact with an item of evidence.

6.8 LR calculations are based on allele frequency estimates from a sampling of each reported population. Uncertainty in these estimates, as well as uncertainties in population composition, F_{ST} , STRmix™ modeling, and MCMC are all accounted for by reporting the HPD with a one-sided quantile of 0.99 and incorporating the factor of $N!$. All reasonable uncertainty is conceded.

7 Calculations

Not applicable.

8 Measurement Uncertainty

Not applicable.

9 Sampling

The examiner will ensure the results in the *Laboratory Report* relate only to the items tested or sampled.

9.1 A reasonable assumption of homogeneity can be made for reference samples and various types of evidence examined by the DNA Units. This type of evidence includes known samples (e.g., blood tubes, buccal samples), bones, teeth, hair and swabs.

9.2 When a reasonable assumption of homogeneity cannot be assumed, the *Laboratory Report* will reflect the tested portion of the item of evidence, making no inference about the whole.

10 Safety

Not applicable.

11 References

FBI Laboratory Quality Assurance Manual

FBI Laboratory Operations Manual

DNA Procedures Manual

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Rev. #	Issue Date	History
3	09/12/18	<p>Edits throughout to incorporate new SWGDAM recommended verbal scale, update report language, and to include Identifiler® Plus reinterpretation guidelines. Clarified and restructured as needed.</p> <p>Figure 2: removed GF ladder picture and renumbered figures 4.3.2, 5.1.1.3, Glossary: removed bleed-through throughout</p> <p>4.3.2.1.b: specified ladder usage</p> <p>4.4 c: changed guidance when the negative control is not as expected</p> <p>5.1.1.1.1: removed estimate calculation; specified that STRmix™ does not use this method</p> <p>5.2.1.5: added reference to report wording for contamination</p> <p>5.4.2: updated determination of number of contributors guidance with greater detail</p> <p>5.4.3: updated sex determination guidance for clarity</p> <p>5.4.7.1: removed redundant paragraph</p> <p>5.4.7.3: updated section to assess whether included POIs fit together in a mixture, and if not, add report language to address</p> <p>5.4.10: loosened requirements for entering profiles into CODIS; updated example to clarify</p> <p>5.5.1.4: added guidance to assess the conditioning of elimination samples in STRmix™ and added example language of elimination samples provided but not used</p> <p>5.5.2.10: new section to incorporate TEDAC language</p> <p>6.3: replaced negative control limitation with close relatives limitation</p> <p>Added references</p> <p>New Appendix E</p>
4	03/16/20	<p>1,4.4.b, 5.2.1.2, 5.4, 5.4.5.1, 5.5.10.3: revised for clarity</p> <p>3: updated GMIDX version</p> <p>5.1.1: Added reference to SOPs for ID+ GMIDX settings</p> <p>5.1.1.1.1: updated stutter table based on additional validation studies</p> <p>5.1.1.1.4: consolidated and updated spike guidance</p> <p>5.1.1.1.6: added additional information re: artifacts</p> <p>5.4.3: new criteria for sex determination implemented</p> <p>5.4.6.1, 5.4.9.2: added reference to Appendix F.</p> <p>5.4.7.3.4: removed “in the mixture” to make applicable to single source profiles</p> <p>5.4.8.2: added separate tri-allele guidance for evidentiary and reference samples</p> <p>5.5: added reference for DNA procedure</p> <p>5.5.1.1, 5.5.4: language updated for Trio implementation</p> <p>5.5.1.2: added example when a previous reference sample was reported, added additional examples</p> <p>5.5.1.4: removed “at this time” from the example language</p> <p>5.5.2.4: corrected and clarified based on our practice</p> <p>5.5.2.8: added sampling description statement</p>

Rev. #	Issue Date	History
		5.5.9: removed requirement to add a statement when no results are entered into CODIS
		9: added reporting guidance for sampling
		11: added a reference
		Appendix A, Figures 2 and 3: added statement about adjusting Pt
		Appendix A, 7: new criteria for sex determination to mirror 5.4.3
		Appendix E, changed title to Interpretation, added reference to 229, added requirement for authorization to reinterpret, moved ID+ STRmix settings to Appendix F

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 03/13/2020

DCU Chief

Date: 03/13/2020

SBAU Chief

Date: 03/13/2020

Appendix A: GlobalFiler™ GMIDX Analysis Settings

Analysis Method Editor

General **Allele** Peak Detector Peak Quality SQ & GQ Settings

Bin Set: GlobalFiler_Bins_v1

☒ Use marker-specific stutter ratio and distance if available

Marker Repeat Type:		Tri	Tetra	Penta	Hexa
Global Cut-off Value		0.0	0.0	0.0	0.0
MinusA Ratio		0.1	0.1	0.0	0.0
MinusA Distance	From	0.75	0.75	0.0	0.0
	To	1.25	1.25	0.0	0.0
Global Minus Stutter Ratio		0.0	0.0	0.0	0.0
Global Minus Stutter Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0
Global Plus Stutter Ratio		0.0	0.0	0.0	0.0
Global Plus Stutter Distance	From	0.0	0.0	0.0	0.0
	To	0.0	0.0	0.0	0.0

Amelogenin Cutoff 0.0

Range Filter... Factory Defaults

Save As Save Cancel Help

Figure 1 - Allele Tab for GlobalFiler™ 150AT and 50AT

Appendix A: GlobalFiler™ GMIDX Analysis Settings (cont.)

The screenshot shows the 'Analysis Method Editor' dialog box with the 'Peak Detector' tab selected. The dialog is divided into several sections for configuring peak detection parameters.

General: The 'Peak Detection Algorithm' is set to 'Advanced'.

Ranges: This section contains two sub-sections: 'Analysis' and 'Sizing'.
- **Analysis:** 'Partial Range' is selected in the dropdown. 'Start Pt' is 3450 and 'Stop Pt' is 8800.
- **Sizing:** 'Partial Sizes' is selected in the dropdown. 'Start Size' is 60 and 'Stop Size' is 460.

Smoothing and Baseline:
- **Smoothing:** Radio buttons for 'None', 'Light' (selected), and 'Heavy'.
- **Baseline Window:** Set to 33 pts.

Size Calling Method: Radio buttons for '2nd Order Least Squares', '3rd Order Least Squares', 'Cubic Spline Interpolation', 'Local Southern Method' (selected), and 'Global Southern Method'.

Peak Detection:
- **Peak Amplitude Thresholds:** B: 150, R: 150, G: 150, P: 150, Y: 150, O: 150.
- **Min. Peak Half Width:** 2 pts.
- **Polynomial Degree:** 3.
- **Peak Window Size:** 13 pts.
- **Slope Threshold:** Peak Start: 0.0, Peak End: 0.0.

Normalization: A checkbox labeled 'Use Normalization, if applicable' is checked.

Buttons: At the bottom are 'Save As', 'Save', 'Cancel', and 'Help'. A 'Factory Defaults' button is located above the 'Save' button.

Figure 2 - Peak Detector Tab for GlobalFiler™ 150AT
Note: Analysis “Start Pt” and “Stop Pt” may be adjusted as needed

Appendix A: GlobalFiler™ GMIDX Analysis Settings (cont.)

The screenshot shows the 'Analysis Method Editor' dialog box with the 'Peak Detector' tab selected. The dialog is divided into several sections for configuring peak detection parameters.

General: The 'Peak Detection Algorithm' is set to 'Advanced'.

Ranges: This section contains two sub-sections: 'Analysis' and 'Sizing'.
- 'Analysis' has a dropdown menu set to 'Partial Range', with 'Start Pt' at 3350 and 'Stop Pt' at 8250.
- 'Sizing' has a dropdown menu set to 'Partial Sizes', with 'Start Size' at 60 and 'Stop Size' at 460.

Smoothing and Baseline: This section includes 'Smoothing' options (None, Light, Heavy) with 'Light' selected, and a 'Baseline Window' set to 33 pts.

Size Calling Method: This section includes radio button options for '2nd Order Least Squares', '3rd Order Least Squares', 'Cubic Spline Interpolation', 'Local Southern Method' (selected), and 'Global Southern Method'.

Peak Detection: This section includes 'Peak Amplitude Thresholds' for B (50), R (50), G (50), P (50), Y (50), and O (150). It also includes 'Min. Peak Half Width' (2 pts), 'Polynomial Degree' (3), and 'Peak Window Size' (13 pts).

Slope Threshold: This section includes 'Peak Start' and 'Peak End' both set to 0.0.

Normalization: This section includes a checkbox for 'Use Normalization, if applicable' which is checked.

Buttons: At the bottom right is a 'Factory Defaults' button. At the bottom are 'Save As', 'Save', 'Cancel', and 'Help' buttons.

Figure 3 - Peak Detector Tab for GlobalFiler™ 50AT

Note: Analysis “Start Pt” and “Stop Pt” may be adjusted as needed

Appendix B: GlobalFiler™ STRmix™ Analysis Settings

STRmix - Default Settings

Default Settings

MCMC settings	Inputs and Outputs	Likelihood Ratio
<input type="text" value="3"/> # MCMC chains	<input type="checkbox"/> Extended Output	<input type="text" value="1000"/> HPD iterations
<input type="text" value="500000"/> MCMC accepts	<input type="text" value="20"/> Alleles per locus	<input type="text" value="99.0"/> Sig value
<input type="text" value="100000"/> Burnin accepts	Summary:	<input type="text" value="1"/> Sides
<input type="text" value="9.0"/> Post burn-in shortlist	<input checked="" type="checkbox"/> Analysis	<input checked="" type="checkbox"/> Factor of N! LR
<input type="text" value="0.005"/> Random Walk SD	<input checked="" type="checkbox"/> LR	<input checked="" type="checkbox"/> Include MCMC uncertainty
<input type="checkbox"/> Low Memory Mode	<input checked="" type="checkbox"/> Parameters	
	<input checked="" type="checkbox"/> Weightings	
	<input checked="" type="checkbox"/> Settings	
	<input checked="" type="checkbox"/> Inputs	
	<input checked="" type="checkbox"/> Interpretations	
	Default Kit <input type="text" value="FBI_GlobalFiler"/>	
<input type="text" value="Z:\DSU Users"/> Default Text File Directory <input type="text" value="Z:\DSU Users"/> Default STRmix File Directory		

Cancel Save

Redacted

Figure 1 – STRmix™ V2.4.05 default settings

Appendix B: GlobalFiler™ STRmix™ Analysis Settings (cont.)

STRmix - Add/Edit DNA Profiling Kit

Add/Edit DNA Profiling Kit:

DNA Profiling Kit: **FBI_GlobalFiler**

Kit name: **FBI_GlobalFiler**

Stutter File: **FBI_GlobalFiler_Stutter.txt**

Stutter Exceptions File: **FBI_GlobalFiler_Exceptions.csv**

Forward Stutter File: **FBI_GlobalFiler_Forward Stutter.txt**

Number of Loci: **24** Gender Locus: **AMEL**

Locus Order: **D3S1358,VWA,D16S539,CSF1PO,TPOX,Yindel,AMEL,D8S1179,D21S11,D18S51,DYS391,D2S441,D19S433,TH01,FGA,D22S1045,D5S818,D13S317,D7S820,SE33,D10S1248,D151656,D12S391,D251338**

Include Loci: **Y,Y,Y,Y,N,Y,Y,N,Y,Y,Y,Y,Y,Y,Y,Y,Y**

Detection Threshold: **150,150**

0.3 Stutter max: **0** Drop-in cap: **5.92,1.473** Allelic Variance

0.15 Forward stutter max: **0.0** Drop-in frequency: **5.006,3.474** Stutter Variance

-1.0 Degradation starts at: **0.0** Drop-in parameters: **0.5** Var > mode

0.01 Degradation max: **30000** Saturation: **0.016** Locus Amp Variance

Redacted

Figure 2 – GlobalFiler™ 150AT kit settings

STRmix - Add/Edit DNA Profiling Kit

Add/Edit DNA Profiling Kit:

DNA Profiling Kit: **FBI_GlobalFiler_50AT**

Kit name: **FBI_GlobalFiler_50AT**

Stutter File: **FBI_GlobalFiler_Stutter.txt**

Stutter Exceptions File: **FBI_GlobalFiler_Exceptions.csv**

Forward Stutter File: **FBI_GlobalFiler_Forward Stutter.txt**

Number of Loci: **24** Gender Locus: **AMEL**

Locus Order: **D3S1358,VWA,D16S539,CSF1PO,TPOX,Yindel,AMEL,D8S1179,D21S11,D18S51,DYS391,D2S441,D19S433,TH01,FGA,D22S1045,D5S818,D13S317,D7S820,SE33,D10S1248,D151656,D12S391,D251338**

Include Loci: **Y,Y,Y,Y,N,Y,Y,N,Y,Y,Y,Y,Y,Y,Y,Y,Y**

Detection Threshold: **50,50**

0.3 Stutter max: **150** Drop-in cap: **7.021,1.147** Allelic Variance

0.15 Forward stutter max: **0.003** Drop-in frequency: **3.881,3.866** Stutter Variance

-1.0 Degradation starts at: **0.0** Drop-in parameters: **0.5** Var > mode

0.01 Degradation max: **30000** Saturation: **0.0189** Locus Amp Variance

Redacted

Figure 3 – GlobalFiler™ 50AT kit settings

Appendix B: GlobalFiler™ STRmix™ Analysis Settings (cont.)

FBI GlobalFiler_Reverse Stutter -			FBI GlobalFiler_Forward Stutter -		
File	Edit	Format View Help	File	Edit	Format View Help
Locus, Intercept, Slope			Locus, Intercept, Slope		
1,	-0.03981,	0.00665	1,	0.00677,	0
2,	-0.07490,	0.00797	2,	0.00954,	0
3,	-0.04410,	0.00809	3,	0.01100,	0
4,	-0.04394,	0.00817	4,	0.01017,	0
5,	-0.02449,	0.00475	5,	0.00851,	0
6,	0,	0	6,	0,	0
7,	0.00697,	0.00359	7,	0.00669,	0
8,	-0.04235,	0.00346	8,	0.01132,	0
9,	-0.03495,	0.00636	9,	0.00944,	0
10,	0,	0	10,	0,	0
11,	0.02831,	0.00102	11,	0.00661,	0
12,	-0.04794,	0.00734	12,	0.00877,	0
13,	0.00284,	0.00195	13,	0.00587,	0
14,	-0.05513,	0.00511	14,	0.00770,	0
15,	-0.10177,	0.01140	15,	-0.04565,	0.00473
16,	-0.03532,	0.00771	16,	0.00735,	0
17,	-0.04504,	0.00784	17,	0.00663,	0
18,	-0.03732,	0.00729	18,	0.00777,	0
19,	0.03208,	0.00197	19,	0.00889,	0
20,	-0.04018,	0.00761	20,	0.00735,	0
21,	0.01163,	0.00353	21,	0.00850,	0
22,	-0.07182,	0.00729	22,	0.01125,	0
23,	-0.00353,	0.00328	23,	0.00733,	0

Figure 4 – Parameters used in determination of allele-specific stutter ratios at GlobalFiler™ loci (150AT and 50AT)

Appendix B: GlobalFiler™ STRmix™ Analysis Settings (cont.)

[illegible]

Figure 5 – GlobalFiler™ stutter values included in the Stutter Exceptions File where longest uninterrupted stretch (LUS) information is available

Appendix B: GlobalFiler™ STRmix™ Analysis Settings (cont.)

STRmix - Population Settings

Step 3: Population Settings

FBI_Trinidadian

Add Population

Remove Population

Population	Proportion	FST	Allele Freq File
FBI_Caucasian	0.111111111111111	0.01b(1.0,1.0)	FBI_Caucasian.csv
FBI_Apache	0.111111111111111	0.03b(1.0,1.0)	FBI_Apache.csv
FBI_AA_BAH_JAM	0.111111111111111	0.01b(1.0,1.0)	FBI_AA_BAH_JAM.csv
FBI_Chamorro	0.111111111111111	0.01b(1.0,1.0)	FBI_Chamorro.csv
FBI_Filipino	0.111111111111111	0.01b(1.0,1.0)	FBI_Filipino.csv
FBI_Navajo	0.111111111111111	0.03b(1.0,1.0)	FBI_Navajo.csv
FBI_SEH	0.111111111111111	0.01b(1.0,1.0)	FBI_SEH.csv
FBI_SWH	0.111111111111111	0.01b(1.0,1.0)	FBI_SWH.csv
FBI_Trinidadian	0.111111111111111	0.01b(1.0,1.0)	FBI_Trinidadian.csv

Range

Profiles originates from 1 to 1 contributors

Use MLE for contributor # under Hp and Hd

Stratify contributor #

Factor N!

Display Factor of N! LR

Use informed Mx priors

User informed Mx priors

Sampling Variation

Calculate HPD

Include MCMC uncertainty

HPD iterations: 1000

Quantile: 99

Sides: 1

Save as default

Cancel

Back

Start

Start & Search

Redacted

Figure 6 – STRmix™ V2.4.05 Population options and parameters

Appendix C: Glossary

Adenylation – also referred to as non-template-dependent nucleotide addition. *Taq* DNA polymerase is known to add an additional nucleotide (typically “A”) to the 3' ends of double-stranded PCR products in a non-template-dependent manner. This phenomenon results in the generation of the “plus-A” fragment which is the allele (N) produced by amplification. When an additional nucleotide is not added to a fragment, it results in a “minus-A” fragment, an artifact of the PCR process.

Allelic Ladder – a mixture of common alleles which is run separate from any sample(s) or control(s), and is used by the software as a reference for designating alleles. Each ladder is a kit reagent that consists of amplified allelic fragments of known size and repeat content. The ladder does not contain all possible alleles that could be detected at an individual STR locus (e.g., off-ladder alleles).

Alternate Reference Sample – a specimen of established origin that may be used as a reference sample (e.g., a DNA typing result obtained from blood on the clothing of a victim known to have been bleeding or an item of personal effect).

Analytical Threshold (AT) – the minimum peak height that confidently ascribes a true amplicon peak and the height below which confidence is too low to reliably assign a peak as an allele. The AT is higher than the limit of detection of the system to increase the confidence that any given peak at or above this threshold is a PCR product.

Concordance – agreement in allele calls/DNA types at shared loci between different amplifications of the same sample. Agreement does not require that the allele calls be identical but rather that they were amplified from a common source. Determination of concordance between different amplifications of a sample must take into account kit differences and differences in the quantity of the DNA amplified. Concordance can be also determined for samples creating a composite DNA profile (e.g., different extractions of the same stain or the same bone); however, these determinations must additionally take into account differences resulting from sampling and extraction (e.g., efficiency of the differential).

Conditional Reference Sample (i.e., assumed contributor) – the reference sample from an individual that corresponds to an evidence item taken from an anatomical location (e.g., vaginal swab, oral swab, fingernail clippings) or item of direct physical contact (e.g., under shorts, panties, bra) which is expected to yield DNA from the individual from whom the specimen was taken.

Contamination – the unintentional introduction of foreign DNA into a sample. Can be categorized as **random** or **systemic**.

Daughter Plate – the plate containing samples that have been prepared for CE. Prepped samples usually consist of a mixture of formamide, size standard, and amplified DNA.

Differential Extraction – a type of extraction that procedurally subdivides the sample into male and female fractions. The female/non-sperm/F fraction is enriched for DNA of non-sperm origin such as epithelial cells and white blood cells. The male/sperm/M fraction is enriched for DNA of sperm cell origin.

Disassociated Primer Dye – a dye that has become detached from its primer resulting in non-specific, reproducible peaks. These peaks may be specific to a given lot of amplification kits.

Discordance – Non-agreement in allele calls/DNA types at shared loci between different amplifications of purportedly the same sample that cannot be explained by differences in the amplification kit used and/or differences in the quality or quantity of the DNA amplified. Discordance indicates that the results were from different sources. Discordance may be also determined between different extractions of the same stain or the same bone.

Distinguishable Mixture – a mixture of DNA in which donors contributed different amounts of biological material to the sample, resulting in the ability to attribute alleles to an individual donor(s).

Dropout – the failure to detect an allele(s) in a sample usually due to stochastic amplification of low levels of DNA.

Extraction Control – contains all of the chemical solutions used in the analysis process except any DNA containing sample and is processed through the same extraction, quantitation, amplification, and electrophoretic typing procedures as the evidentiary specimens. An extraction control monitors aspects of the analytical processes for the introduction of adventitious DNA.

F_{ST} – the chance that two alleles from two different people are identical by descent.

GeneScan®-500 Internal Size Standard (GS-500) – consists of DNA fragments of known sizes (35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490, and 500 bp) that are labeled with an orange dye (GS-500 LIZ), and are combined with an aliquot of each amplified sample during daughter plate preparation prior to electrophoresis. This standard is used to size peaks that are detected by the CE instrument that are differentiated with blue, green, yellow, and red fluorescent dyes. Due to the temperature sensitivity of the 250 bp fragment's sequence-based conformation, this fragment is usually not used for sizing purposes.

GeneScan®-600 Internal Size Standard Version 2 (GS-600v2) – consists of DNA fragments of known sizes (20, 40, 60, 80, 100, 114, 120, 140, 160, 180, 200, 214, 220, 240, 250, 260, 280, 300, 314, 320, 340, 360, 380, 400, 414, 420, 440, 460, 480, 500, 514, 520, 540, 560, 580 and 600bp) that are labeled with an orange dye (GS-600 LIZ), and are combined with an aliquot of each amplified sample during daughter plate preparation prior to electrophoresis. This standard is used to size peaks that are detected by the CE instrument that are differentiated with blue, green, yellow, and red and purple fluorescence dye. Due to the temperature sensitivity of the 250 bp fragment's sequence-based conformation, this fragment is usually not used for sizing purposes.

Indistinguishable Mixture – a typing result from a sample for which alleles cannot be attributed to individual donors, which occurs when similar amounts of biological material are contributed to the specimen by multiple donors.

Local Southern Method – used for size calling samples amplified with the GlobalFiler™ Kit. This method uses the four fragments closest in size to the unknown fragment to determine a best fit line. This best fit line is an average of two such curves created by using three standard points each (the first makes use of the two points below and one point above the unknown fragment and the other one point

below and two points above). In this way, only the region of the size standard near the fragment(s) of unknown length is analyzed.

Major Contributor – the component of a distinguishable mixture who donated the preponderance of DNA. When using STRmix™, a major contributor is the contributor with the highest mixture proportion of DNA, which is at least double that of the next contributor's DNA proportion.

MCMC – Markov Chain Monte Carlo; an algorithm based on standard mathematical principles to assign weights to genotype combinations.

Microvariant – an allele that contains an incomplete repeat unit.

Minor Contributor – the component of a mixture who donated a lesser amount of DNA in relation to the major contributor.

Minus A – a product of the amplification process which results in a peak that is one base pair shorter than the allelic fragment due to the failure to add a nucleotide to the 3' end. The height of the –A peak is generally <15% of the allelic peak. It is more likely to be observed when excessive template DNA amounts are used in the PCR and/or when an inhibitor of *Taq* DNA Polymerase is present in the sample.

Negative Amplification Control – contains all of the chemical components required for the amplification of DNA, with the exception of any DNA sample or extraction control. It is processed through the same amplification and electrophoretic typing procedures as any sample(s), and is used to monitor aspects of the analytical processes for the introduction of adventitious DNA that may have occurred during or after amplification.

Off-ladder Allele (OL) – DNA fragments of genetic origin that fail to size within a defined allele category (e.g., a bin or virtual bin).

Person of Interest (POI) – the person for which you are determining the weight of the evidence. The POI can be the subject, the victim, or any individual for whom you have a reference sample.

Positive Amplification Control – contains all of the chemical components required for the amplification of DNA including a known DNA source (e.g., 9947A or 007), and serves as a general qualitative indicator of amplification, as well as a confirmation that the software has functioned accurately in assigning alleles.

Proposition – describes the two hypotheses being compared. One hypothesis is that an individual is included as a contributor to the evidence along with additional unknown, unrelated individual(s), as needed. The other hypothesis is that the evidence originates from unknown, unrelated individual(s). In some instances, an assumed contributor may be included in both hypotheses.

Pull-up – a low intensity peak that derives from excessive fluorescence intensity of another peak and generally observed in the color(s) spectrally adjacent to the high intensity peak.

Raised Baseline – appears in an electropherogram as a non-specific elevation of the horizontal axis between one or more peaks. This elevation can result from excessive template DNA or be instrument related (e.g., a misaligned capillary).

Random Contamination – incidents of adventitious DNA that occur in a manner that suggests that the source of extraneous DNA was introduced into a sample(s) or control(s) and was not a part of a material and/or a reagent common to all of the samples processed as a batch. Extremely low level contamination, such as a result at the amelogenin locus with no results at any STR loci, may also be considered random. Additionally, if one of multiple reagent blanks within a batch was contaminated, the conclusion of random contamination may be supported by demonstrating that another reagent blank from the batch was not contaminated. (e.g., by an equally or more sensitive amplification, if no signal was indicated during quantitation).

Spike – non-specific, non-reproducible peak(s) that may result during electrophoresis from electrical fluctuations in the power source, from the interference of urea crystals, bubbles, or inherently fluorescent materials such as detergent in the capillary.

Stochastic Threshold – an empirically determined parameter that specifies the minimum peak height that all allelic peaks at a given locus must display to be confident that no genetic components of a sample failed to be detected due to differential amplification (i.e., stochastic loss due to low template mass, degraded template DNA, and/or PCR inhibition). This parameter is established for each amplification kit.

Stutter – phenomenon inherent to the PCR amplification of repetitive DNA sequences, and often is presented as a minor PCR product peak one repeat unit smaller (e.g., N-4) than the source allelic peak (N). Stutter peaks originate *in vitro* (i.e., the PCR reaction) from an allele that exists *in vivo* (i.e., the template DNA) and do not constitute an individual's genotype. The size and intensity of stutter peaks are generally predictable based on the alleles that are detected in a specimen.

STRmix™ – a software system that applies a fully continuous probabilistic genotyping approach to DNA profile interpretation. It standardizes the analysis of profiles within a laboratory by using estimates of variance of electropherograms derived from the laboratory's own DNA profiling data.

Systemic Contamination – incidents of adventitious DNA that occur in a manner that suggests that the source of the introduced DNA was a material and/or a reagent common to some or all of the samples processed as a batch. It is noted that systemic contamination may not affect all samples equally and may be present in some but not all of the samples in the batch. Additionally, contamination may be systemic to an extraction batch without affecting the other extraction batches in an amplification batch.

Trace Contributor – a contributor whose alleles exhibit low amplitude, as well as possible allele dropout and/or imbalance of heterozygous alleles due to stochastic amplification.

Tri-allele – a rare anomaly resulting in three alleles at a locus in a single individual.

Virtual Bin – an allele sizing category designated by the software that has no corresponding allele physically present in the ladder.

Appendix D: *Allele frequency data for Minnesota Native American Population*

GlobalFiler™ (Life Technologies, Inc.) data (i.e., fsa files) for the Minnesota Native American population samples were generated by the Minnesota Bureau of Criminal Apprehension and provided to the FBI Laboratory DNA Support Unit (DSU). Genotyping was performed using GeneMapper® ID-X software version 1.4 (Life Technologies, Inc.). Microsoft Excel was used to calculate allele frequencies. Arlequin version 3.5.2.2 (Excoffier 2010) was used to test the hypothesis that none of the loci departed from Hardy-Weinberg equilibrium ($p > 0.05$). Three of 21 markers yielded a p value < 0.05 ; however, after applying the Bonferroni correction, the affected loci were found to also be in equilibrium. This dataset is suitable in terms of both size and quality for the purposes of estimating DNA profile probabilities. It is noted that N is equal to the number of alleles typed.

Minnesota Native American Population Globalfiler Expanded STR Loci Allele Frequencies																								
Allele	D3S1358	vWA	D16S539	CSF1PO	TPOX	Y indel	D8S1179	D21S11	D18S51	DYS391	D2S441	D19S433	Th01	FGA	D22S1045	D5S818	D13S317	D7S820	SE33	D16S1248	D1S1656	D12S391	Allele	
2						1.000000																	2	
6													0.195846										6	
7					0.003378								0.442968			0.165541							7	
8			0.006757	0.006757	0.405405		0.006757						0.037162				0.057432	0.128378					8	
9			0.141892	0.087838	0.049319		0.003378			0.213115	0.016892		0.077703			0.057432	0.179054	0.081081					9	
9.3													0.243243										9.3	
10			0.162162	0.320946	0.030405		0.050676		0.010135	0.426230	0.277027					0.047297	0.128378	0.209459					10	
11	0.003378		0.290541	0.020703	0.388314		0.027027			0.344262	0.442568	0.003378			0.094595	0.391892	0.226351	0.256892		0.027027	0.034014		11	
11.3											0.020270												11.3	
12		0.003401	0.263514	0.327703	0.128378		0.125000		0.131757	0.016393	0.037162	0.033784			0.003378	0.236486	0.253378	0.277027	0.010135	0.013514	0.061224		12	
13		0.003401	0.008108	0.043919		0.351351		0.091216			0.023649	0.168919				0.097973	0.067568	0.037162		0.287162	0.098639		13	
13.2												0.158784											13.2	
14	0.064489	0.040816	0.027027	0.010135		0.310811		0.239919			0.165541	0.239865			0.013514	0.003378	0.084459		0.010135	0.334459	0.068027		14	
14.2												0.040541											14.2	
14.3																							14.3	
15	0.418919	0.074830				0.101351		0.128378		0.016892	0.141892				0.405405	0.003378			0.016892	0.212838	0.142857	0.010135	15	
15.2												0.108108											15.2	
15.3																							15.3	
16	0.310811	0.340136					0.016892		0.111486			0.037162			0.429054				0.033784	0.108108	0.221088	0.010135	16	
16.2												0.023649											16.2	
16.3																					0.047619		16.3	
17	0.108108	0.323129				0.006757			0.125000			0.006757			0.030405				0.037162	0.013514	0.054422	0.040541	17	
17.2												0.027027											17.2	
17.3																					0.153061	0.010135	17.3	
18	0.084459	0.156463				0.030405		0.030405						0.010135	0.016892		0.091216	0.003378	0.003401	0.273649	0.060811		18	
18.2												0.010135				0.006757							18.2	
18.3																0.003378				0.074830	0.003378		18.3	
19	0.010135	0.040816					0.040541		0.040541					0.101351	0.006757		0.074324				0.216216	0.307432	19	
19.2																						0.027027	19.2	
19.3																							19.3	
20	0.013605								0.020270						0.128378				0.057432			0.135135	0.091216	20
20.2																			0.003378				20.2	
21	0.003401						0.006757		0.006757					0.128378			0.040541	0.004541		0.094595	0.027027		21	
21.2																	0.013514						21.2	
22							0.006757		0.006757					0.108108			0.016892		0.067568	0.145270			22	
22.2														0.003378		0.0023649							22.2	
23							0.003378							0.148649	0.020270		0.020270		0.091216	0.128378			23	
23.2																0.006757							23.2	
24														0.185811					0.016892	0.087838			24	
24.2																		0.013514					24.2	
25														0.128378					0.003378	0.030405			25	
25.2																	0.027027						25.2	
26														0.043919									26	
26.2														0.003378									26.2	
27						0.006757								0.006757				0.070946					27	
27.2																		0.064189					27.2	
28						0.064189													0.057973				28	
28.2																							28.2	
29						0.185811																	29	
29.2																		0.087973					29.2	
30						0.327703																	30	
30.2						0.023649													0.101351				30.2	
31						0.067568																	31	
31.2						0.185811													0.043919				31.2	
32						0.003378																	32	
32.2						0.081081													0.006757				32.2	
33						0.003378																	33	
33.2						0.040541																	33.2	
34.2						0.010135													0.010135				34.2	
Allele	D3S1358	vWA	D16S539	CSF1PO	TPOX	Y indel	D8S1179	D21S11	D18S51	DYS391	D2S441	D19S433	Th01	FGA	D22S1045	D5S818	D13S317	D7S820	SE33	D16S1248	D1S1656	D12S391	Allele	

Appendix E: *Interpretation of Identifiler® Plus data*

When making conclusions, the reevaluation of any of the allele calls or genotype calls, removal of alleles (or entire loci) from statistical estimates, or a change in the assumptions is considered reinterpretation. The DNA examiner must be previously qualified in the interpretation of data from the legacy amplification kit and platform instrument model and authorized to perform reinterpretation of legacy data.

1. GMIDX settings

The GMIDX software settings are represented in the procedure for interpretation of results from the Identifiler® Plus amplification kit (i.e., DNA 229) and/or the procedures for interpretation of legacy DNA data (i.e., DNA 230).

2. Non-Specific Peaks

Non-specific peaks are defined as peaks of unknown origin. Those known to be associated with specific commercial products are noted below.

- a. Non-specific peaks of multiple colors have been observed in some lots of the GS-500. Because the GS-500 is added to each sample in preparation for electrophoresis, these non-specific peaks that originate from the GS-500 can be seen in any sample.
- b. Excessive non-specific peaks are defined as peaks of unknown origin at two or more loci that result in stretches of non-specific, low amplitude data. Samples exhibiting excessive non-specific peaks may have been re-injected for reduced time(s) to facilitate interpretation.

3. Non-Human Peaks

The amplification of non-human DNA may exhibit characteristics such as:

- a. A peak at ~98 bp (before amelogenin) and/or randomly at other loci.
- b. A peak at D21S11 called a “28.2”.
- c. Alleles absent at the majority of the locations but not consistent with a degraded profile or low level human DNA sample

Non-human DNA typing results should not be used for comparisons.

Appendix E: Interpretation of Identifiler® Plus data (cont.)

4. Stutter

The Identifiler® Plus (27 or 28 cycles) stutter percentage guidelines provided in Table 4 are estimates (Average + 3 SD) of the maximum expected relative negative stutter, or N-4, values Positive stutter, or N+4, can also occur, and is typically less than 5% for all loci.

Locus	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539
% Stutter	11	12	10	11	13	6	12	11

Locus	D2S1338	D19S433	vWA	TPOX	D18S51	D5S818	FGA
% Stutter	13	13	13	7	15	11	13

Table 1 – Maximum Expected Stutter Percentage Guidelines

5. Application of Peak Height Thresholds to Allelic Peaks

5.1 The analytical threshold (AT) is 50 RFU.

5.2 The stochastic thresholds (ST) are:

- 200 RFU for the Identifiler® Plus (27 cycles) Amplification Kit
- 300 RFU for the Identifiler® Plus (28 cycles) Amplification Kit

6. Peak Height Ratio

The values provided in Table 2 are estimates for the minimum expected PHR percentages for the AmpF/STR® Identifiler® Plus (27 cycles) Amplification Kit and are based on the average PHRs observed in the validation studies. The PHR guidelines are only applicable to allelic peaks that meet or exceed the ST.

Peak Height	All Identifiler® Plus STR Loci (27 cycles)
200-499 RFU	50%
500-999 RFU	60%
1000 RFU and above	70%

Table 2 – Minimum Expected Heterozygous Peak Height Ratio Guidelines (3130xl Data)

Appendix E: *Interpretation of Identifiler® Plus data (cont.)*

7. DNA Typing Results Subjected to STRmix™ Interpretation

7.1 STRmix™ analysis may be conducted on evidentiary typing results amplified using the Identifiler® Plus Kit with 27 cycles and separated on the Applied Biosystems 3130XL for STRmix™ analysis. Reference profiles are not constrained by these requirements for STRmix™ analysis.

7.2 Typing results assumed to originate from 1 to 4 contributors may be interpreted using STRmix™. In addition, if conditioned on at least one contributor, a 5-person mixture may be interpreted using STRmix™. Five-person mixtures without an assumed contributor, as well as mixtures of DNA from more than 5 contributors, will not be interpreted using STRmix™, and will be reported as unsuitable for comparisons.

8. STRmix™ Settings and Usage

8.1 STRmix™ software settings for versions 2.3.06 and 2.4.05, including FBI specific parameters from internal validation studies, are depicted in Appendix F.

8.2 The latest version of STRmix™ validated for Identifiler® Plus Kit will generally be used for analysis.

8.3 If STRmix™ was previously run and statistics reported for a person of interest (POI), the LR from previous (LRFP) function will be used for analysis of additional POIs. The LRFP function is restricted to the version of STRmix™ used for the original deconvolution. If the original deconvolution is not available, it will be recreated in STRmix™ by setting the seed in the version used for the original deconvolution.

8.4 If STRmix™ was previously run solely for the purposes of a CODIS deconvolution, the LRFP function may be used for analysis of POIs submitted at a later time. Alternatively, a new deconvolution using the latest version of STRmix™ may be performed.

9. Unsuitable Results

Five-person mixtures without an assumed contributor, as well as mixtures of DNA from more than 5 contributors, will be reported as unsuitable for analysis.

Appendix F: *Identifiler® Plus STRmix™ Analysis Settings*

Default Settings

MCMC settings	Inputs and Outputs	Likelihood Ratio
# MCMC chains: 8	Extended output: N	HPD iterations: 1000
MCMC accepts: 500000	Alleles per locus: 20	Sig value: 99.0
Burnin accepts: 100000	Summary:	Sides: 1
Post burn-in shortlist: 9.0	<input checked="" type="checkbox"/> Analysis	<input checked="" type="checkbox"/> Factor of N! LR
Random Walk SD: 0.005	<input checked="" type="checkbox"/> LR	<input checked="" type="checkbox"/> Include MCMC uncertainty
HR range: 10000.0	<input checked="" type="checkbox"/> Parameters	
	<input checked="" type="checkbox"/> Weightings	
	<input checked="" type="checkbox"/> Settings	
	<input checked="" type="checkbox"/> Inputs	
	<input checked="" type="checkbox"/> Interpretations	
	Default Kit: FBI_IdentifilerPlus	

Text file dir default: C:\Users\rsjust\Desktop

STRmix file dir default: C:\Users\rsjust\Desktop

Cancel Save

Redacted

Figure 1 – STRmix™ V2.3.06 default settings
(brefer to Appendix B Figure 1 for STRmix™ V2.4.05 default settings)

Appendix F: *Identifiler® Plus STRmix™ Analysis Settings (cont.)*

STRmix - Add/Edit DNA Profiling Kit

Add/Edit DNA Profiling Kit

DNA Profiling Kit:

Kit name:

Stutter File:

Stutter Exceptions File:

Forward Stutter File:

Number of Loci: Gender Locus:

Locus Order:

Include Loci:

Detection Threshold:

<input type="text" value="0.3"/>	Stutter max	<input type="text" value="0"/>	Drop-in cap	<input type="text" value="4.2818,1.0671"/>	Allelic Variance
<input type="text" value="0.0"/>	Forward stutter max	<input type="text" value="0.0"/>	Drop-in frequency	<input type="text" value="9.1442,1.1239"/>	Stutter Variance
<input type="text" value="-1.0"/>	Degradation starts at	<input type="text" value="0,0"/>	Drop-in parameters	<input type="text" value="0.1"/>	Var > mode
<input type="text" value="0.01"/>	Degradation max	<input type="text" value="7000"/>	Saturation	<input type="text" value="0.0113"/>	Locus Amp Variance

Redacted

Figure 2 – STRmix™ V2.4.05 Identifiler® Plus kit settings

Appendix F: *Identifiler® Plus STRmix™ Analysis Settings (cont.)*

Add/ Edit DNA profiling kit

DNA profiling kit: **FBI_IdentifilerPlus** [Edit Kit] [Delete Kit]

Kit name: **FBI_IdentifilerPlus**

Stutter File: **IdentStutter_FBI.txt** [Find File] [Edit File]

Stutter Exceptions File: **FBI_IdentifilerPlus_Exception.csv** [Find File] [Edit File]

Number of Loci: **16** Gender Locus: **AMEL**

Locus Order: **SF1PO,D3S1358,TH01,D13S317,D16S539,D2S1338,D19S433,WWA,TPOX,D18S51,AMEL,D5S818,FGA**

Include Loci: **Y,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y,Y** [Ignore Loci]

Detection Threshold: **50,50,50,50,50,50,50,50,50,50,50,50,50,50,50** [Set Td]

0.3 Stutter max 0 Drop-in cap 4.2818,1.0671 Allelic Variance

7000 Saturation 0.0 Drop-in frequency 9.1442,1.1239 Stutter Variance

-1.0 Degradation starts at 0,0 Drop-in parameters 0.1 Var > mode

0.01 Degradation max 0.0113 Locus Amp Variance

[Cancel] [Save Kit]

Redacted

Figure 3 - STRmix™ V2.3.06 Identifiler® Plus kit settings

Appendix F: *Identifiler® Plus STRmix™ Analysis Settings (cont.)*

Locus	Intercept	Slope
1	0.0087	0.00378
2	-0.0725	0.0046
3	-0.0446	0.00868
4	-0.0565	0.00971
5	-0.0446	0.00773
6	-0.0102	0.00409
7	-0.046	0.00829
8	-0.0529	0.00948
9	-0.00363	0.00369
10	-0.0672	0.00987
11	-0.0595	0.00752
12	-0.0243	0.00587
13	-0.0155	0.00594
14	-0.0442	0.00884
15	-0.0357	0.00468

Figure 4 – Parameters used in determination of allele-specific stutter ratios at Identifiler® Plus loci

Appendix F: *Identifiler® Plus STRmix™ Analysis Settings (cont.)*

Allele	D8S1179	D21S11	D7S820	CSF1PO	D3S1358	TH01	D13S317	D16S539	D2S1338	D19S433	vWA	TPOX	D18S51	D5S818	FGA
5	0	0	0	0	0	0.0072	0	0	0	0	0	0	0	0	0
6	0	0	0	0	0	0.01258	0	0	0	0	0	0	0	0	0
7	0.0289	0	0	0	0	0.01796	0	0	0	0	0.03207	0	0	0	0
8	0.0351	0	0	0	0	0.02334	0	0	0	0	0	0	0	0	0
8.3	0	0	0	0	0	0.0072	0	0	0	0	0	0	0	0	0
9	0.0413	0	0	0	0	0.02872	0	0	0	0	0	0	0	0	0
9.3	0	0	0	0	0	0.01258	0	0	0	0	0	0	0	0	0
10	0.0475	0	0	0	0	0.0541	0	0	0	0.03318	0	0	0	0	0
10.3	0	0	0	0	0	0.01796	0	0	0	0	0	0	0	0	0
11	0.0537	0	0	0	0.03143	0.03948	0	0	0	0.04209	0.03207	0	0	0	0
11.3	0	0	0	0	0	0.02334	0	0	0	0	0	0	0	0	0
12	0.0599	0	0	0	0.04012	0	0	0	0	0.051	0	0	0	0	0
12.1	0	0	0	0	0	0	0	0	0	0.00645	0	0	0	0	0
12.2	0	0	0	0	0	0	0	0	0	0.05991	0	0	0	0	0
13	0.0537	0	0	0	0.04881	0	0	0	0	0.05991	0.03207	0	0	0	0
13.2	0	0	0	0	0	0	0	0	0	0.06882	0	0	0	0	0
14	0.0599	0	0	0	0.06619	0	0	0	0	0.06882	0.035675	0	0	0	0
14.2	0	0	0	0	0	0	0	0	0	0.07773	0	0	0	0	0
15	0.0661	0	0	0	0.070535	0	0	0	0.05184	0.07773	0.057305	0	0	0	0
15.2	0	0	0	0	0	0	0	0	0	0.08664	0	0	0	0	0
16	0.0661	0	0	0	0.079225	0	0	0	0.05834	0.08664	0.064515	0	0	0	0
16.2	0	0	0	0	0	0	0	0	0	0.09555	0	0	0	0	0
17	0.0661	0	0	0	0.087915	0	0	0	0.06484	0.09555	0.06812	0	0	0	0.04038
17.2	0	0	0	0	0	0	0	0	0	0.10446	0	0	0	0	0
18	0.0785	0	0	0	0.09226	0	0	0	0.07134	0.10446	0.071725	0	0	0	0.04572
18.2	0	0	0	0	0	0	0	0	0	0.11337	0	0	0	0	0
19	0	0	0	0	0.10095	0	0	0	0.06809	0	0.08254	0	0	0	0.05106
19.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0564
20	0	0	0	0	0.10964	0	0	0	0.06484	0	0.08975	0	0	0	0.0564
20.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.06174
21	0	0	0	0	0	0	0	0	0.06484	0	0.09696	0	0	0	0.06174
21.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.06708
21.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.0297
22	0	0	0	0	0	0	0	0	0.07134	0	0.10417	0	0	0	0.06708
22.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.07242
23	0	0	0	0	0	0	0	0	0.07784	0	0	0	0	0	0.07242
23.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.07776
24	0	0	0	0	0	0	0	0	0.08434	0	0	0	0	0	0.07776
24.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.07776
24.2	0	0.0443	0	0	0	0	0	0	0	0	0	0	0	0	0.0831
25	0	0.0592	0	0	0	0	0	0	0.09084	0	0	0	0	0	0.0831
25.2	0	0.0592	0	0	0	0	0	0	0	0	0	0	0	0	0.08844
26	0	0.0443	0	0	0	0	0	0	0.09734	0	0	0	0	0	0.08844
26.2	0	0.06665	0	0	0	0	0	0	0	0	0	0	0	0	0
27	0	0.049217	0	0	0	0	0	0	0.10384	0	0	0	0	0	0.07776
28	0	0.055475	0	0	0	0	0	0	0.11034	0	0	0	0	0	0.09912
28.2	0	0.0592	0	0	0	0	0	0	0	0	0	0	0	0	0
29	0	0.062925	0	0	0	0	0	0	0	0	0	0	0	0	0.07242
29.2	0	0.0592	0	0	0	0	0	0	0	0	0	0	0	0	0
30	0	0.06665	0	0	0	0	0	0	0	0	0	0	0	0	0
30.2	0	0.0592	0	0	0	0	0	0	0	0	0	0	0	0	0
31	0	0.070875	0	0	0	0	0	0	0	0	0	0	0	0	0
31.2	0	0.06665	0	0	0	0	0	0	0	0	0	0	0	0	0
32	0	0.08155	0	0	0	0	0	0	0	0	0	0	0	0	0
32.2	0	0.079017	0	0	0	0	0	0	0	0	0	0	0	0	0
33	0	0.089	0	0	0	0	0	0	0	0	0	0	0	0	0
33.2	0	0.079017	0	0	0	0	0	0	0	0	0	0	0	0	0
34	0	0.08155	0	0	0	0	0	0	0	0	0	0	0	0	0
34.2	0	0.089	0	0	0	0	0	0	0	0	0	0	0	0	0
35	0	0.06665	0	0	0	0	0	0	0	0	0	0	0	0	0
35.2	0	0.09645	0	0	0	0	0	0	0	0	0	0	0	0	0
36	0	0.0741	0	0	0	0	0	0	0	0	0	0	0	0	0
36.2	0	0.1039	0	0	0	0	0	0	0	0	0	0	0	0	0
37.2	0	0.089	0	0	0	0	0	0	0	0	0	0	0	0	0
38.2	0	0.08155	0	0	0	0	0	0	0	0	0	0	0	0	0
39.2	0	0.08155	0	0	0	0	0	0	0	0	0	0	0	0	0

Figure 5 – Identifiler® Plus stutter values included in the Stutter Exceptions File where LUS information is available

Appendix F: *Identifiler® Plus STRmix™ Analysis Settings (cont.)*

Step 3: Population Settings

FBI_Trinidadian

Add Pop

Del Pop

Change Fst

	Population	Proportion	Fst	Allele Freq File
1	FBI_AA_BAH_JAM	0.1	0.01b(1.0,1.0)	FBI_AA_BAH_JAM
2	FBI_Apache	0.1	0.03b(1.0,1.0)	FBI_Apache.csv
3	FBI_Caucasian	0.1	0.01b(1.0,1.0)	FBI_Caucasian.c
4	FBI_Chamorro	0.1	0.01b(1.0,1.0)	FBI_Chamorro.c
5	FBI_Filipino	0.1	0.01b(1.0,1.0)	FBI_Filipino.csv
	FBI_MN_Native_Am	0.1	0.03b(1.0,1.0)	FBI_MN_Native_
	FBI_Navajo	0.1	0.03b(1.0,1.0)	
	FBI_PU	0.1	0.01b(1.0,1.0)	

Range

Profiles originates from 1 to 1 contributors

☐ Use MLE for contributor # under Hp and Hd
☒ Stratify contributor #

Factor N!

☒ Display Factor of N! LR

Use informed Mx priors

☐ User informed Mx priors

Sampling Variation

☒ Calculate HPD
☒ Include MCMC uncertainty

HPD iterations

1000

Quantile

99

Sides

1

Save as default

Cancel

Back

Start

Start & Search

Redacted

Figure 6 – STRmix™ V2.3.06 Population options and parameters for calculation of the LR (refer to Appendix B Figure 6 for STRmix™ V2.4.05 options and parameters)

FDDU

Procedures for Receiving Collection Kits and Sample Plate Preparation

1 Scope

These procedures apply to DNA personnel who perform sample receipt or check in, submission management, plate creation, plate preparation, and/or punch collection kits received by the Federal DNA Database Unit (FDDU). The FDDU may receive three types of collection kits: a buccal collection kit; a liquid blood collection kit, which contains a liquid blood tube; or a finger stick collection kit, which contains dried bloodstain card(s).

2 Equipment/ Materials/ Reagents

Equipment/Materials

- General Laboratory Supplies
- STACS (Sample Tracking and Control System) Software (STACS DNA Inc.) version 6.0 or above
- Barcode printer with appropriately sized labels (2.0" x 0.5" or equivalent)
- Barcode Scanner (Honeywell or equivalent)
- 96-Well Sample (MicroAmp) Plates (aka PCR Plate) (Applied BioSystems or equivalent)
- Plate Sealer, microplate (Agilent PlateLoc or equivalent) with heat seal
- Optical Adhesive Covers (Applied BioSystems or equivalent)
- RFID Tags, Reader and software (VISI-TRAC RFID or equivalent)
- Bloodstain card (Whatman® FTA® Genecard or equivalent)
- Sample storage pouches (Fitzco or equivalent)
- Agilent Robotic Workstation (Agilent Bravo)
- Agilent Vworks Software, version 11.2 or higher
- Punch Instrument (BSD600-Duet, BSD 600Plus, or equivalent)

Reagents

- SwabSolution™ Kit (Promega)
- Prep-N-Go Buffer (Applied Biosystems)
- Water, Reagent Grade (VWR or equivalent)
- Buffer, Low TE (aka TEKnova DNA Suspension Buffer) (Fisher Scientific or equivalent)

3 Standards and Controls

Two Combo controls (aka Negative) and two Blood/Buccal Internal Standard (BIS) controls are included on each amplification plate.

The BIS is prepared as described in the DNA QA procedure for reagents (i.e., DNAQA 609) and is added to the plate during the sample punch procedure. These controls will be interpreted according to the criteria in the applicable FDDU Procedure (i.e., FDDU 315).

4 Procedures

4.1 Receipt of Sample Collection Kits

FDDU collection kits are shipped to the FBI Laboratory via US Postal Service Business Reply Mail and, on occasion, by an express mail service. The kits are delivered to the FDDU by FBI Laboratory mailroom personnel or picked up from the mailroom by FDDU personnel.

4.1.1 Using STACS, record kits received by the FDDU by scanning the FDDU collection kit barcode affixed to the collection kit. If necessary, barcodes may be hand-entered.

4.1.1.1 If kits are not logged into STACS on the same day as received, the date received should be recorded on a collection kit, batch, or bin, as appropriate, until they are logged into STACS.

4.1.1.2 When all received kits have been scanned each batch or bin of kits, as appropriate, should be labeled with the date received.

4.1.2 Proceed to **Check In and Barcoding** or place the FDDU collection kits in storage. Store kits containing buccal or dried bloodstain samples at room temperature and kits containing blood tubes refrigerated.

Place kits containing barcoded samples into storage at room temperature. Place each storage container containing barcoded blood tubes into refrigerated storage. Store the FD-936 forms in a designated area.

4.2 Check In and Barcoding of Collection Kits

Retrieve kits to be checked in. Kits should be processed in order of the date received. Each person performing the check in procedures must only have one kit open at a time and process the kits individually.

4.2.1 Scan the FDDU collection kit barcode. Inspect the kit for integrity issues (e.g. damaged packaging or lacking a tamper-evident seal). Integrity issues may result in a kit being marked as unacceptable.

4.2.2 Open the kit. Each kit must contain the following:

- a. Completed FD-936 form.
- b. FDDU Sample(s) (e.g., buccal collection device, blood sample in an EDTA

vacutainer tube, dried bloodstain card(s))

4.2.3 Ensure that the same subject name or alternate unique identification number (e.g., FBI#, SSN, Alien#, BOP#, FINS#, collection device/card barcode(s)) is on the FD-936 and the corresponding sample.

4.2.4 STACS may auto-populate the following fields; however, the user may manually enter or change the entries, if necessary.

- Contributor Type
- Submitting Agency
- Specimen Nature
- Count

4.2.5 Determine if the kit is deemed acceptable. If not, select a reason from the pull-down menu. If an applicable reason is not available in the pull-down menu, “Other” may be selected and the reason entered in the appropriate field. Check in and barcoding is completed for all kits; however, unacceptable kits will be evaluated according to applicable FDDU Procedure (i.e. FDDU 302).

4.2.6 Additional information may be entered into the comment field, if necessary.

4.2.7 Upon completion of the sample check in, STACS prints a set of barcodes. Place the appropriate barcode labels on the FD-936 and each sample. Ensure that the barcode label placed on the FD-936 corresponds to the barcode label placed on the respective sample(s).

4.2.8 Place a RFID tag on each sample.

4.2.9 Scan the STACS barcodes and the RFID tags to associate them in STACS.

***NOTE:** If necessary, the RFID software may be used to associate the sample(s) to the RFID tag(s).*

4.2.10 Place the sample(s) (e.g., buccal cards, blood cards, buccal cassettes) into a sample storage pouch for storage. Place blood tubes in the designated storage location.

4.2.11 Collection devices (if provided) may be properly discarded following check in of the collection kit.

Repeat above steps to check in each additional kit.

4.2.12 Place all packaged samples into an appropriate storage container (e.g., box or plastic sleeve) labeled with an RFID container tag.

4.2.13 Verify the contents of each storage container using an RFID reader. If necessary, resolve any discrepancies regarding the inventory of the container.

4.2.14 Place each storage container containing barcoded samples into storage at room temperature. Place each storage container containing barcoded blood tubes into refrigerated storage. Store the FD-936 forms in a designated area.

4.3 Blood Spotting

The FDDU occasionally receives liquid blood samples, which must be manually dried onto a bloodstain card (e.g., FTA Genecard) prior to initiating the DNA analysis procedure.

4.3.1 Prior to spotting a liquid blood sample, the corresponding FTA card must be labeled with the appropriate STACS barcode and RFID tag.

4.3.2 Retrieve each blood tube from refrigerated storage and allow to come to room temperature. Invert the blood tube several times before processing.

4.3.3 Scan the matching STACS barcodes affixed to the blood tube and FTA card.

4.3.4 Spot approximately 50 µl of the liquid blood onto each circle on the FTA card in a laminar flow hood.

4.3.5 Discard the liquid blood tube in the appropriate biohazard container.

4.3.6 Allow the bloodstain card to dry for approximately 1 hour.

4.3.7 Place the bloodstain card and a desiccant pouch into a sample storage pouch.

4.3.8 Place all packaged bloodstain cards into an appropriate storage container labeled with an RFID container tag.

4.3.9 Place each storage container containing barcoded bloodstain cards into storage at room temperature or in a freezer.

4.4 Plate Creation

All accepted samples are available for processing based on the selection of the amplification kits. Blood samples may be analyzed using the GlobalFiler Express amplification kit. Buccal samples may be analyzed with the Identifiler Direct or GlobalFiler Express amplification kits. Once an amplification kit is selected, the sample(s) are available to be selected for a plate and processed in the laboratory.

4.4.1 Select and gather the appropriate samples to be allocated to the plate.

4.4.2 STACS may prompt the user to populate the following fields as appropriate:

- a. Plate Cycle Number – 25, 26, 27, or 28
- b. Plate Punch Size – 1.2 mm

4.4.3 Upon completion of plate creation, STACS prints out unique plate barcodes. Place each barcode accordingly, on the PCR plate and support base.

4.4.4 Scan the barcodes affixed to both the PCR plate and support base. STACS verifies the scanned barcodes.

4.5 Plate Preparation

Refer to DNA Procedure Introduction (DNA QA 600) for applicable laboratory quality assurance and cleaning instructions.

4.5.1 Select appropriate plates to be processed and indicate whether the plate preparation process will be done manually or using the Agilent Bravo. If using the Agilent Bravo, load the plates onto the instrument deck.

4.5.2 Scan the barcodes affixed to each plate and the reagent(s) required for the selected scenario. If using the Agilent Bravo, scan the instrument, click process in STACS and follow prompts from associated VWorks software.

4.5.3 Add appropriate anti-static reagent to the plate(s), if required.

- a. Identifiler Direct - Add 2µl of reagent grade water. This combination may be performed manually or using the Agilent Bravo Robotic Workstation.
- b. GlobalFiler Express (FTA Paper) - Add 3µl of DNA Suspension Buffer (Low TE Buffer). This combination may be performed manually or using the Agilent Bravo Robotic Workstation.
- c. GlobalFiler Express (Non-FTA Paper) - Add 3µl of Prep-N-Go Buffer. This combination may be performed manually or using the Agilent Bravo Robotic Workstation.
- d. GlobalFiler Express (FTA Paper and Non-FTA Paper) – Add 3µl of SwabSolution. This combination may be performed manually or using the Agilent Bravo Robotic Workstation.

NOTE: Alternatively, for Identifiler Direct Plate Prep, 4µl of reagent grade water, 2µl of Prep-N-Go buffer, or no anti-static reagent are also suitable. These may be added manually or using the Agilent Bravo Robotic Workstation.

4.5.4 Select whether the plate preparation process was successful, failed, or aborted. Comments and observations should be entered for plates with process failed or aborted results.

4.6 Sample Punch

The following summarizes the combinations validated for sample processing:

Sample Type	Punch Size	Amplification Kit	Amplification Cycles
Blood	1.2mm	GlobalFiler Express	26 or 28
Buccal	1.2mm	Identifiler Direct	25, 26, 27 or 28
		GlobalFiler Express	26 or 28

4.6.1 Ensure necessary cleaning procedures are performed on the punch instrument prior to use.

4.6.2 Select a created plate.

4.6.2.1 Scan and punch the samples or controls into each of the allocated wells on the punch instrument in the order displayed in STACS.

NOTE: When punching samples on a BSD, ensure that the required “Cleaning Strike(s)” will be placed in between each sample by using the appropriate designation in the BSD Configuration file. Punch the “cleaning sample(s)” (e.g., clean FTA card, clean filter paper) when prompted.

4.6.3 Upon completion of punching a plate:

- Visually inspect the plate to verify its integrity.
- Indicate the result as successful, failed, or aborted.
- Comments and observations must be entered for plates with process failed results.

4.6.4 Cover successfully punched plates and transfer them to the laboratory for processing.

5 Sampling

A reasonable assumption of homogeneity can be made for database samples; therefore, any sampling (i.e., punch) will be considered representative of the entire sample.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

Only the following combinations are approved for sample processing:

Sample Type	Plate Prep	Punch Size	Amplification Kit	Amplification Cycles
Blood (FTA)	3ul of DNA Suspension Buffer (Low TE)	1.2mm	GlobalFiler Express	26 or 28
Buccal (FTA)	Nothing or 2ul or 4ul water or 2ul Prep-N-Go	1.2mm	Identifiler Direct	25, 26, 27 or 28
	3ul of DNA Suspension Buffer (Low TE)	1.2mm	GlobalFiler Express	26 or 28
	3ul of SwabSolution	1.2mm	GlobalFiler Express	26
Buccal (Non-FTA)	3ul of Prep-N-Go Buffer	1.2mm	GlobalFiler Express	26 or 28
	3ul of SwabSolution	1.2mm	GlobalFiler Express	26

9 Safety

9.1 All FDDU samples that contain blood are considered potentially infectious regardless of the perceived status of the source individual or the age of the material. All FDDU personnel who work with such material will follow the “Bloodborne Pathogen Exposure Control Plan” found in the most current version of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Safe Work Practices and Procedures”, “Bloodborne Pathogen Exposure Control Plan”, “Personal Protective Equipment”, and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual* for important personal safety information.

9.3 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

10 References

FBI Laboratory Quality Assurance Manual.

FBI Laboratory Operations Manual.

FBI Laboratory Safety Manual.

DNA Procedures Manual.

STACS DNA Inc. *Sample Tracking and Control System (STACS) User's Guide*.

Symbol Technologies Inc. *Symbol Barcode Reader User's Guide*.

Zebra Technologies International. *Zebra Barcode Printer User's Guide*.

BSD600-Duet Semi-Automated Dried Sample Punch Instrument Operator Guidelines (BSD Robotics)

Rev. #	Issue Date	History
5	03/11/16	Revised entirety of document for simplification and clarity and to remove software interface instructions. Added procedures for plate punch previously in 304-7.
6	12/09/16	Revised to incorporate GlobalFiler Express and non-FTA buccal samples. Changed cards and sample cards to samples throughout.
7	02/28/18	Changed STACS version to 6.0 Removed instructions for the Wallac punch instrument and Identifiler amplification kit throughout. Changed 4°C to refrigerated and -20°C to frozen throughout. 4.3: Added blood spotting instructions, renumbered remaining sections.
8	09/13/19	Added SwabSolution procedures throughout. 1 Update scope 2 Added BSD 600Plus 3 Added info to Standards and Controls 4.1 Added how samples are transported to and receive by the lab. 4.2.5 Added additional info for unaccepted kits. 4.4.1 Relocated gathering samples. 4.4.2 Removed 24 cycle option missed on Rev 7 and punch count. 5 Added Sampling info. 8 Updated table.

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 09/12/2019

FDDU Chief

Date: 09/12/2019

QA Approval

Quality Manager

Date: 09/12/2019

FDDU Procedures for Processing and Management of Submission Information

1 Scope

These procedures apply to DNA personnel who process the Request for National DNA Database Entry (FD-936) form data included in each Federal DNA Database Unit (FDDU) collection kit and manage sample submission and status information.

2 Equipment/Materials/Reagents

- **Redacted**
- Document Scanner (Kodak i2900 Scanner or equivalent)
- Laserfiche Software (Version 10.4 or above)
- Laserfiche Quick Fields Software (Version 10.3 or above)
- OCR/ICR Software (TurboScan NG or equivalent)
- STACS (Sample Tracking and Control System) Software (STACS DNA Inc.), version 6.4 or above

3 Procedures

3.1 Processing of FD-936

3.1.1 Processing FD-936 Forms with TurboScan OCR/ICR Software

3.1.1.1 Retrieve forms to be processed. Data entered on an FD-936 is captured and prepared for import using the Laserfiche and/or TurboScan NG Document Capture System. Saved image(s) may be defined as a batch once scanned.

- a. If necessary, utilize the Enhancement module in the TurboScan NG software to fix any imperfections in the images. This step may be automated by the software.
- b. If necessary, utilize the AutoIndex module in the TurboScan NG software to manually process the data. This step may be automated by the software.

3.1.1.2 Use the Turboscan Verification module to verify and edit the fields that were recognized by the TurboScan NG software, as needed. Upon completion of the verification of the batch, save.

3.1.1.3 Use the Turboscan Export module to export the data and images obtained from TurboScan NG. This step may be automated by the software.

3.1.1.4 Using STACS, select appropriate batch for the *Default TurboScan Directory* and *Default File Images Directory* and import the data.

3.1.1.5 Review the data for each submission.

3.1.1.5.1 Make any necessary modifications in STACS or the TurboScan export file.

- If edits are made in STACS, re-validate the data and ensure appropriate corrections have been made.
- If edits are made to the file, save the edited file and close Import window in STACS. Re-import the TurboScan file and ensure appropriate corrections have been made.

3.1.1.5.2 Save the file in STACS.

3.1.1.6 If the submission is valid and contains no errors, the samples will proceed to the laboratory to be processed.

3.1.1.6.1 If the submission is identified as any of the following, additional attention is required. Refer to the appropriate section of this document to manage the submission.

- Missing Information
- Pending Duplicate
- Be On the Look Out (BOLO)
- Pending Reject

3.1.2 Processing FD-936 Forms with Quick Fields and Laserfiche Software

3.1.2.1 Retrieve forms to be processed. Data entered on an FD-936 is captured and prepared for import using the Quick Fields and Laserfiche software.

3.1.2.2 Use the Quick Fields software to review the processing summary of the scanned forms and review/edit the fields flagged by the software.

3.1.2.3 Use the Laserfiche software to export the data and images obtained from Quick Fields. This step may be automated by the software.

3.1.2.4 Using STACS, select appropriate number of submissions and import the data.

3.1.2.5 Review the data for each submission.

3.1.2.5.1 Make any necessary modifications in STACS or Laserfiche.

- If edits are made in STACS, re-validate the data and ensure appropriate corrections have been made.

- b. If edits are made in Laserfiche, save the edited data and close Import window in STACS. Re-export the Laserfiche data for the submission, re-import into STACS and ensure appropriate corrections have been made.

3.1.2.5.2 Save the data in STACS.

3.1.2.6 If the submission is valid and contains no errors, the samples will proceed to the laboratory to be processed.

3.1.2.6.1 If the submission is identified as any of the following, additional attention is required. Refer to the appropriate section of this document to manage the submission.

- Missing Information
- Pending Duplicate
- Be On the Look Out (BOLO)
- Pending Reject

3.2 Submission and Status Information

Once the submissions have been imported into STACS, the image becomes available to view and changes may be made to the data fields for a submission, as necessary. STACS records and tracks all changes.

3.3 Missing or Invalid Information Submissions

3.3.1 STACS identifies a submission as having missing or invalid information based on criteria defined within the software. Some fields may be flagged by STACS if the submission contains missing or invalid information (e.g., Submitting Agency, Contributor type, Subject name, Date of Birth, Identification Number).

3.3.1.1 Missing information submissions are managed or resolved using any of the three available actions:

- a. Issue a request to the submitting agency for the missing information
- b. Enter the missing information
- c. Override the Missing Information status of the submission

3.4 Pending Duplicate Submissions

3.4.1 STACS identifies a submission as a pending duplicate based on criteria defined within the software. Redacted

3.4.1.1 Review the matching fields to determine if the pending duplicate is an actual duplicate. If there are multiple pending matches, the status (e.g., CODIS Confirmed, Pending Reject, Expunged, STR Complete, In Process) should be used as a ranking factor when determining which submission should be selected to confirm a duplicate.

3.4.1.2 If additional information is required to resolve the status of the pending duplicate, contact the submitting agency or query an agency specific system **Redacted** as appropriate.

3.4.1.3 A pending duplicate may be resolved by marking it as an original submission or as a confirmed duplicate to an existing submission. DNA analysis is conducted on all original submissions and may be conducted on confirmed duplicates based on the status of the matching submission.

3.5 Pending Reject Submissions

3.5.1 A pending reject may be identified in STACS if:

- The kit was determined to be unacceptable at sample check in
- The submission was rejected from the Submission Import work list
- A sample retake request was approved
- A missing specimen alert was generated and approved to request another submission

3.5.1.1 Pending rejects may be managed or resolved using any of the three available actions:

- a. Issue a request to the submitting agency for a new sample
- b. Reject the submission
- c. Activate the submission

3.5.1.2 The status of a pending reject submission may be changed for a subset of samples based on rejection reason or for an individual submission.

3.6 BOLO Submissions

3.6.1 If necessary, STACS can create an alert with special instructions for a Be On the Look Out (BOLO) submission.

3.6.1.1 A BOLO may be created by entering the available subject information **Redacted** as well as marking submission(s) with the appropriate instructions (e.g., Do Not Process, High Priority Sample, Normal Priority Sample).

3.6.1.2 STACS searches the database to determine whether a BOLO entry is a potential match to an existing submission or a new submission. If a match is found, the BOLO entry can be changed to a received status in the software and the submission may then proceed to the appropriate module.

3.7 Expedite and Sample Status Requests

3.7.1 Expedite requests and/or sample status requests may be completed upon receipt of appropriate documentation from the requesting agency. STACS allows the user to record and respond to these types of information requests. A response letter may be generated, as needed.

3.7.1.1 If expedite request(s) are received, the sample(s) and/or plate may be marked as high priority. A reason must be entered in the comments field when setting the sample(s) or plate as high priority.

4 Standards and Controls

Not applicable.

5 Sampling

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

Not applicable.

9 Safety

Not applicable.

10 References

DNA Procedures Manual.

FBI Laboratory Quality Assurance Manual.

STACS DNA Inc. *Sample Tracking and Control System (STACS) User's Guide.*

Symbol Technologies Inc. *Symbol Barcode Reader User's Guide.*

Lason Inc. *TurboScan NG User's Guide.*

Rev. #	Issue Date	History
4	06/26/2014	Updated materials in section 3. Deleted cleaning procedures of Turboscan scanner in section 7.2. Revised scanning procedure in section 7.4.1.1. Deleted categories qualifying as Missing Information from Section 7.4.4. Deleted need to include date in comments for entering missing information in section 7.4.6.4. Updated individual to review override of missing information status in sections 7.4.7, 7.4.12, and 7.4.13. Added section 7.4.8.11 and renumbered remainder of section. Updated procedure for Same Status forms in sections 7.4.14.1 and 7.4.14.2. Revised citation of all outside protocol references throughout entire document. Deleted references to FCO Program throughout entire document. Deleted Appendices A-C and all associated references throughout entire document, and renamed remainder of Appendices. Updated references.
5	03/11/2016	Revised entirety of document for clarity and simplification and to remove software interface interactions.
6	01/15/2020	1: Updated the scope to apply to DNA personnel. 2: Updated to STACS 6.4 and changed acronym from STaCS to STACS throughout. 3.1: Updated Processing of FD-936 section to reflect addition of Laserfiche to the TurboScan workflow and the use of Quick Fields and Laserfiche software to scan and process the JABS version of the FD-936 form. 3.4 & 3.5: Deleted potential from section titles and text to reflect STACS module titles. 3.4.1.1: Added Expunged.

Approval

Redacted - Signatures on File

FDDU Chief

Date: 01/14/2020

DNA Technical Leader

Date: 01/14/2020

FDDU Procedures for STR Amplification

1 Scope

These procedures apply to DNA personnel who perform direct amplification of nuclear DNA with the polymerase chain reaction (PCR) using the AmpF/STR® Identifiler® Direct Amplification Kit or the GlobalFiler® Express PCR Amplification Kit on DNA databasing samples received by the Federal DNA Database Unit (FDDU).

2 Equipment/Materials/Reagents

Equipment/Materials

- General Laboratory Supplies
- STACS (Sample Tracking and Control System) Software (STACS DNA Inc.), version 6.0 or higher
- Barcode printer with appropriately sized labels (2.0" x 0.5" or equivalent)
- Barcode Scanner, Hand-held (Symbol LS4000i, 4008i, LS4071 or equivalent)
- Plate Sealer, microplate (Agilent PlateLoc or equivalent) with Heat Sealing Foil
- Optical Adhesive Covers (Applied BioSystems or equivalent)
- Tecan Robotic Workstation (Master Mix Addition)
 - Tecan EVOware Software, version 2.0 or higher (Tecan)
- Optical Cover Compression Pads (Applied BioSystems or equivalent)
- Thermal Cycler (Applied BioSystems GeneAmp® PCR System 9700 or ProFlex™ PCR System)
- Plate centrifuge (Eppendorf Model 5804 or equivalent)
- Laboratory incubator (Isotemp or equivalent)

Reagents

- Liquinox™ Critical Cleaning Liquid Detergent (Alconox or equivalent)
- Water, reagent grade (VWR or equivalent)
- Bleach, 3% (household or equivalent)
- 9947A, 10 ng/μl (Promega DD1001, DD100A or equivalent)
- AmpF/STR® Identifiler® Direct PCR Amplification Kit (Applied BioSystems)
 - Contains AmpF/STR® Identifiler® Direct Master Mix (includes AmpliTaq Gold® enzyme) and AmpF/STR® Identifiler® Direct Primer Set
- GlobalFiler® Express PCR Amplification Kit (Applied BioSystems)
 - Contains GlobalFiler® Express Master Mix, Master Mix Additive, GlobalFiler® Express Primer Set, GlobalFiler® Express Allelic Ladder, and DNA Control 007

3 Standards and Controls

Two Combo controls (aka negative) and two Blood/Buccal Internal Standard (BIS) controls are included on each amplification plate.

The BIS is prepared as described in the DNA QA procedure for reagents (i.e., DNAQA 609) and is added to the plate during the sample punch procedure. These controls will be interpreted according to the criteria in the applicable FDDU procedure (i.e., FDDU 315).

4 Procedures

Refer to the DNA Procedure Introduction (i.e., DNAQA 600) for applicable laboratory quality assurance and cleaning instructions.

When using a Robotic Workstation, ensure general instrument cleaning and maintenance is done prior to use, as needed. See Appendix A for additional guidance.

4.1 Processing with SwabSolution

For plates prepared using SwabSolution, perform the following steps any time prior to loading the plate onto the Tecan instrument or before manually adding master mix to the plate.

- 4.1.1 Ensure that the plate is adequately sealed after punching, then quick-spin the 96-Well plate(s) in a centrifuge (approximately 30 seconds).
- 4.1.2 Incubate the plate(s) at 70°C for 10 minutes.
- 4.1.3 After incubation, quick-spin the 96-Well plate(s) again in a centrifuge (approximately 30 seconds) before proceeding to Master Mix addition.

4.2 Master Mix (MM) Addition

The Master Mix Addition procedure may be performed based on the amplification kit and sample type as follows:

	Identifiler Direct MM	GlobalFiler Express MM
Blood	N/A	Manual or Automated
Buccal	Manual or Automated	

- 4.2.1 Prior to the first use of a GFE kit, prepare the GFE Master Mix tube by adding Master Mix Additive to the GFE Master Mix tube.
 - For a 200 reaction kit: Add 80µL of Additive
 - For a 1000 reaction kit: Add 390µL of Additive

NOTE: Amplification WILL FAIL without the Master Mix Additive in the GFE Master Mix.

- 4.2.2** Prepare the amplification master mix for the required number of samples. Include extra samples in the calculation for overage. Ensure the preparations of the amplification master mix have been recorded in the *Chemical Preparation* module of STACS.

Identifiler Direct MM		GlobalFiler Express MM	
	Per Sample (µl)		Per Sample (µl)
IDD Primer Set	12.5	GFE Primer Set	6.0
IDD Master Mix/Enzyme	12.5	GFE Master Mix w/Additive	6.0

- 4.2.3** Within STACS, select plate(s) to be processed and select the appropriate scenario.

- 4.2.3.1** Additionally, for automated processing only:

- Scan the instrument barcode on the Robotic Workstation.
- Ensure the Robot Maintenance Checks have been performed.
- Indicate whether each check passed.

- 4.2.4** If not previously performed during the selection of the plate(s), scan the barcode on each of the 96-Well plate(s). Scan the barcode of each reagent required for the selected scenario. Select "*Process*" and proceed with the Master Mix Addition procedure.

- 4.2.4.1** Additionally, for automated processing only:

- STACS launches the robotic software
- If necessary, enter the appropriate user name and password at the robotic software log-in screen
- Verify that the appropriate Master Mix Addition script has been opened.
- Ensure the required reagents and the selected 96-Well plate(s) have been loaded on to the instrument. On the Tecan EVO, the plates are loaded in the hotels with well A1 in the back right corner.
- Ensure the instrument has been properly flushed and no air bubbles are visible in the tubing or syringes
- Initiate the robotic software process.
- Indicate the number of plates to be processed.
- Start the Master Mix Addition script.

The following Master Mix Addition procedure will be performed manually or by the Robotic Workstation:

- 4.2.5** Add the required reagents to the appropriate wells in the 96-Well plate(s):

4.2.5.1 For Identifier Direct:

1. Add 25 µL of Identifier Direct master mix to each allocated well.
 - o IDD Ladder wells do not receive master mix.

4.2.5.2 For GlobalFiler Express:

- Add 12 µL of GlobalFiler Express Amplification master mix to each allocated well.
- o GFE Ladder wells do not receive master mix.

4.2.6 For automated processing, the Robotic Workstation will heat-seal the 96-Well plate(s) with a foil cover and centrifuge each plate(s) at 1000 RPM for 1 minute. For manual processing, heat-seal the 96-Well plate(s) with a foil cover or seal the plate with an optical cover.

4.2.7 Visually inspect the plate(s) and indicate the result in STACS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results. If the plate(s) were processed on a Robotic Workstation, indicate in STACS whether the bleach process was performed.

4.3 Identifier® Direct , or GlobalFiler® Express PCR Amplification

4.3.1 If necessary (i.e., manual Master Mix Addition), quick-spin the 96-Well plate(s) in a centrifuge (approximately 30 seconds).

4.3.2 Load each plate into a thermal cycler and place an ABI Optical Compression Pad on each plate and close the thermal cycler(s) lid(s).

4.3.3 Select the appropriate method on the thermal cycler. Available methods are:

Identifier Direct PCR Amplification Method (on 9700)			
	Temperature (C)	Time	Sample Type and Cycles
Hold	95	11 minutes	
Cycle	94	20 seconds	Buccal (25-28 cycles)
	59	2 minutes	
	72	1 minute	
Hold	60	25 minutes	
Hold	4	Forever (∞)	

GlobalFiler Express PCR Amplification Method (on 9700 or ProFlex)			
	Temperature (C)	Time	Sample Type and Cycles
Hold	95	1 minute	
Cycle	94	3 seconds	Buccal or Blood (26 or 28 cycles)
	60	30 seconds	
Hold	60	8 minutes	
Hold	4	Forever (∞)	

- 4.3.4** Start the thermal cycler.
- **Identifiler Direct:** Ensure the reaction volume is 25 µL and the ramp speed is 9600 on a 9700.
 - **GlobalFiler Express:** Ensure the reaction volume is 15 µL and the ramp speed is MAX on a 9700 or GeneAmp™PCR System 9700 simulation mode on a ProFlex.
- 4.3.5** Ensure the *Thermal Cyclers Bar Code* and the *Plate Bar Code* for each plate to be amplified has been scanned into STACS.
- 4.3.6** Indicate the result of the process in STACS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results.
- 4.3.7** After amplification, store plate(s) refrigerated in the post-amplification laboratory at 4°C ± 3°C.

NOTE: The MicroAmp support base used to transport the 96-Well plate from the pre-amplification laboratory space to the post-amplification laboratory space must be cleaned with a 10% bleach solution and/or with 70% isopropyl alcohol prior to or immediately upon return to pre-amplification laboratory space. This practice will minimize the transport of amplified DNA product from post-amplification laboratory space to pre-amplification space.

5 Sampling

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

The appropriate processing methods are selected for a plate based on the sample type added to the plate and the amplification kit to be used. Based on internal studies, only the combinations of processes listed below are approved for use.

Sample Type	Amp Kit	MM Addition	Punch Size	Thermal Cycler	Cycles
Buccal (FTA)	Identifiler Direct	Manual or Automated using a Tecan EVO Robotic Workstation	1.2mm	9700	25, 26, 27 or 28
Buccal (FTA or Non-FTA) or Blood (FTA)	GlobalFiler Express	Manual or Automated using a Tecan EVO Robotic Workstation	1.2mm	9700 or ProFlex	26 or 28

9 Safety

9.1 All FDDU samples that contain blood are considered potentially infectious regardless of the perceived status of the source individual or the age of the material. All FDDU personnel who work with such material will follow the “Bloodborne Pathogen Exposure Control Plan” found in the most current version of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Safe Work Practices and Procedures”, “Bloodborne Pathogen Exposure Control Plan”, “Personal Protective Equipment”, and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual* for important personal safety information prior to conducting these procedures.

9.3 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

10 References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

Federal Bureau of Investigation. Quality Assurance Standards for DNA Databasing Laboratories, current version.

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FBI Laboratory Safety Manual.

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Wang et al. Development and Validation of the AmpFlSTR® Identifiler® Direct PCR Amplification Kit: Multiplex Assay for the Direct Amplification of Single-Source Samples, *Journal of Forensic Science* (2011) doi: 10.1111/j.1556-4029.2011.01757.x.

Rev. #	Issue Date	History
8	03/11/16	Complete rewrite of procedure for simplification, elimination of redundancy and to remove software interface instructions. Added FTA extraction steps from 304-7. Moved QA/QC guidance to Appendices.
9	12/09/16	Updated to incorporate implementation of GlobalFiler Express and Non-FTA samples. Simplified Kit QC set-up instructions in Appendix B.
10	09/13/19	Deleted Identifiler, Agilent BioCel, Tecan TE-MO and FTA procedures throughout. Added Proflex ThermalCycler and corresponding parameters. Added SwabSolution Procedures. Appendix A: Removed Ethanol Wash.

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 09/12/2019

FDDU Chief

Date: 09/12/2019

QA Approval

Quality Manager

Date: 09/12/2019

Appendix A: Instrument Maintenance

Refer to the DNA procedure for equipment calibration and maintenance for additional information and applicable frequency requirements.

1. Robotic Workstations

A. Tecan Instrument Cleaning and General Maintenance Guidance

- **Seal Integrity Check** - Verify that the tubing and syringes (plunger lock screws) are tight and no air bubbles are being introduced into the system.
- **Tip Check** - Verify that the LIHA tips (as appropriate) are tight, free of clogs and not dripping system liquid (RO purified water).
- **ROMA Check** - Verify that the Tecan ROMA(s) and LIHA (as appropriate) are moving and working properly.
- **System liquid level check** - Check and refill the system liquid (i.e., water), if necessary. When refilled, the system liquid should be allowed to de-gas overnight.
- **Clean tips with alcohol** - Clean the outside of the fixed Tecan tips with 70% isopropyl alcohol each workday before use and if they become visually soiled.
- **Bleach deck** - Decontaminate the Tecan workdeck with 10% Bleach each workday before use and if it becomes visually soiled. The barcode scanners should be cleaned with a lint-free cloth only, as needed.
- **System liquid flush** - Flush the instrument thoroughly until no air bubbles are visible in the tubing or syringes.
- **RoboScrub** - Weekly, as used, The Tecan Robotic workstations that utilize the Liquid Handling Arm (LIHA) should be flushed with a mild detergent (e.g., Liquinox) to clean the inside of the Tecan tips, tubing and syringes to maintain the precision and accuracy of liquid handling by the Tecan Robotic Workstation. The RoboScrub solution should remain in the Tecan for a minimum of 15 minutes. Following the elapsed time, flush the Tecan with water until there are no visible air bubbles in the tubing or syringes
- **Bleach process** - Following each run on the Tecan EVO workstations, decontaminate the inside and outside of the Tecan tips with 3% Bleach and flush with water. There is a Bleach Wash script that will perform this process.

B. Performance Verification (aka Artel)

An Artel MVS (Multichannel Verification System) and NIST traceable standards are used to test the accuracy and precision of the liquid handling by a Tecan Robotic Workstation. Refer to the Artel MVS Multichannel Verification System User Guide for operation of the Artel MVS.

For the LIHA (typically configured with eight (8) fixed tips), perform a minimum of 6 repetitions with each tip for each volume.

The accuracy and precision results must be within the tolerance limits set by the FDDU for each volume.

- At times, it may be necessary to modify/optimize the Tecan liquid class parameters (e.g., offset and factor) to ensure that the accuracy and precision of the Tecan continually meets the FDDU tolerance limits.
- The tolerance limits, Artel data, and any modifications to Tecan liquid classes must be maintained in a notebook or an electronically accessible file/folder for reference.

2. Thermal Cycler General Maintenance and Performance Verification

A. 9700

Refer to the *GeneAmp®* PCR System 9700 User's Manual Set 96-Well Sample Block Module User's Manual for instructions on how to perform the following procedures.

- **Cleaning** - Refer to *Cleaning the sample wells* and *Cleaning the sample block cover* sections.
Temperature Verification Test - This test procedure requires the use of a Temperature Verification System (Applied Biosystems) and verifies that the thermal cycler remains within the temperature accuracy specification. Refer to *Running the Calibration Verification Test* section.
Temperature Non-uniformity Test - This test procedure requires the use of a Temperature Verification System and verifies the temperature uniformity of the sample wells in the thermal cycler. Refer to *Running the Temperature Non-uniformity Test* section.
- **Rate Test and Cycle Test** - These procedures verify the integrity of the cooling and heating system of a thermal cycler. Refer to *Running System Performance Diagnostics* section.

B. ProFlex™

Refer to the ProFlex™ PCR System User Guide for instructions on how to perform the following procedures.

- **Cleaning** – Refer to the chapter for maintaining the instrument for instructions on how to clean the sample wells and heated cover.
- **Verify Block Temperature** – These tests are found in the *Block Verification Test* screen and requires the use of a Temperature Verification System for the following test types:
 - **Heated Cover Test** - This test verifies the proper functioning of the heated cover.
 - **Temperature Verification Test** - This procedure verifies that the thermal cycler remains within the temperature accuracy specification.
 - **Temperature Non-Uniformity Test** - This procedure verifies the temperature uniformity of the sample wells in the thermal cycler.

Appendix B: QC of Critical Reagents

Identifiler Direct Kit

Each new lot of Identifiler Direct Kits will be evaluated by testing it jointly with the current lot using the following samples per lot, at minimum:

- 5 - Punches (1.2mm) of a BIS (buccal) control,
- 1 - Combo control,
- 5 - 9947A controls with a template quantity of 4 ng

Amplify the plate at 25 cycles using the Identifiler Direct protocol.

Inject the plate, with at least 2 allelic ladders per lot, 3x at the current CE settings.

Acceptance Criteria:

The allelic ladders from both the new lot and current lot of kits must be used separately to analyze the QC data with GeneMapper ID-X. Criteria for acceptance of kits for use with FDDU samples are:

1. BIS and 9947A samples:
 - a. Acceptable peak morphology and balance at each locus.
 - b. Average peak heights of all alleles are greater than 150 RFU.
 - c. Correct typing results obtained.
 - d. No allelic peaks, other than those attributable to the BIS and 9947A controls, are detected.
2. Each ladder allele is greater than 100 RFU.
3. Acceptable results for the negative controls.

If all the above criteria are met then the new lot is approved for use and the allelic ladders included in both the new lot and current lot of kits may be used for the analysis of samples both within and between lots of Identifiler Direct kits. Enter the results of the QC procedures in STACS using the *Receiving* modules.

GlobalFiler Express Kit

Each new lot of GlobalFiler Express Kits will be evaluated by testing it jointly with the current lot using the following samples per lot, at minimum:

- 5 - punches (1.2mm) of a BIS (buccal) control
- 1 - Combo control
- 4 - 007 controls with a template quantity of 5 ng

Amplify the plate at 26 cycles using the GlobalFiler Express protocol.

Inject the plate, with at least 2 allelic ladders per lot, 3x at the current CE settings.

Acceptance Criteria:

The allelic ladders from both the new lot and current lot of kits must be used separately to analyze the QC data with GeneMapper ID-X. Data will be analyzed using normalization. Criteria for acceptance of kits for use with FDDU samples are:

1. BIS and 007 samples:
 - a. Acceptable peak morphology and balance at each locus.
 - b. Average peak heights of all alleles are greater than 175 RFU.
 - c. Correct typing results obtained.
 - d. No allelic peaks, other than those attributable to the BIS and 007 controls, are detected.
2. Each ladder allele is greater than 175 RFU.
3. Acceptable results for the negative controls.

If all the above criteria are met then the new lot is approved for use and the allelic ladders included in both the new lot and current lot of kits may be used for the analysis of samples both within and between lots of GlobalFiler Express kits. Enter the results of the QC procedures in STACS using the *Receiving* modules.

FDDU

Procedures for Capillary Electrophoresis using the 3130xl or 3730

1 Scope

These procedures apply to the preparation of daughter plates of amplified samples followed by the separation by capillary electrophoresis (CE) with an AB 3130xl Series Genetic Analyzer or an AB 3730 Series DNA Analyzer in the Federal DNA Database Unit (FDDU).

2 Equipment/Materials/Reagents

Equipment/Materials

- General Laboratory Supplies (e.g., pipettes, tubes)
- Barcode printer with appropriately sized labels (2.0" x 0.5" or equivalent)
- Barcode Scanner, Hand-held (Symbol LS4000i, 4008i, LS4071 or equivalent)
- STaCS (Sample Tracking and Control System) Software (STaCS DNA Inc.), version 5.0 or higher
- Robotic Workstation (Tecan EVO 150/200)
 - Tecan EVOware Software, version 2.0 or higher (Tecan)
- 96-Well Sample (MicroAmp) Plates (Applied BioSystems or equivalent)
- 96-Well Plate Septa (Applied BioSystems or equivalent)
- Plate Sealer, microplate (Agilent Plate Loc or equivalent) with heat sealing plastic
- GeneAmp® PCR System 9700 Thermal Cycler (Applied BioSystems)
- 3130xl Genetic Analyzer (Applied BioSystems)
 - 3130xl Data Collection Software, version 3.0 or higher (Applied BioSystems)
 - 96-Well Plate Base and Retainer (Applied BioSystems or equivalent)
 - Capillary Array (3130xl/3100), 36 cm x 50 µm (Applied BioSystems)
- 3730 DNA Analyzer (Applied BioSystems)
 - 3730 Data Collection Software, version 3.0 or higher (Applied BioSystems)
 - 96-Well Plate Base and Retainer (Applied BioSystems or equivalent)
 - Capillary Array (3730), 36 cm (Applied BioSystems)

Reagents

- AmpF/STR® Identifiler® Allelic Ladder (Applied BioSystems)
- AmpF/STR® Identifiler® Direct Allelic Ladder (Applied BioSystems)
- Hi-Di™ Formamide (Applied BioSystems or equivalent)
- GeneScan 600 LIZ Size Standard (GS-600 [LIZ]) (Applied BioSystems)
- Bleach, 3% (household or equivalent)
- Liquinox™ Critical Cleaning Liquid Detergent (Alconox or equivalent)
- Water, reagent grade (VWR #48218-710 or equivalent)
- 10X Genetic Analyzer Buffer with EDTA (Applied BioSystems or equivalent)
- Performance Optimized Polymer, AB 3130xl POP-4® or AB 3730 POP-7® (Applied BioSystems)
- Matrix Standard Kit, Multi-Capillary DS-33 (Dye Set G5 and G5-RCT) (Applied BioSystems 4345833)

3 Standards and Controls

The following controls are included on each amplification plate using the listed kit and will be transferred for capillary electrophoresis. These controls will be interpreted according to the criteria in the applicable FDDU Procedure.

Identifiler (ID)	Identifiler Direct (IDD)
Negative	Negative (aka Combo)
Amp Blank	BIS
Positive (9947A)	
BIS	

4 Procedures

Refer to DNA Procedure Introduction (DNA QA 600) for applicable laboratory quality assurance and cleaning instructions.

When using a Robotic Workstation, ensure general instrument cleaning and maintenance is done prior to use, as needed. See the FDDU Procedure for FTA Preparation and STR Amplification (FDDU 305) Appendix A for additional guidance.

4.1 Electrophoresis Plate Preparation (EPP) Daughter Plate Creation

- 4.1.1 Create the daughter plate in STaCS.
- 4.1.2 Upon completion of the daughter plate creation, STaCS prints out plate barcodes with a daughter plate designation. Place each barcode accordingly, on the EPP MicroAmp plate and support base.
- 4.1.3 Scan the barcodes affixed to both the EPP MicroAmp plate and support base. STaCS verifies the scanned barcodes.
- 4.1.4 Repeat for each plate that is being processed at EPP.

4.2 GS-600 [LIZ] Formamide Preparation

4.2.1 Prepare the GS-600 [LIZ] formamide. The solution is prepared by combining Hi-Di formamide with GS-600 [LIZ] size standard in a 50:1 ratio. One extra plate should be included in the calculation for overage.

GS-600 [LIZ] Formamide (per 96-Well daughter plate)	
Hi-Di™ Formamide	2500 µl
GS-600 [LIZ] size standard	50 µl

4.2.2 Ensure the preparation has been recorded in the *Chemical Preparation* module of STaCS.

4.2.3 Store the GS-600 [LIZ] formamide in a refrigerator and use within the same day as it was prepared.

4.3 Electrophoresis Plate Preparation (EPP)

The EPP procedure will be performed based on the amplification kit and the sample type:

	Identifiler	Identifiler Direct
Blood	Manual or Automated	N/A
Buccal	Manual	Manual or Automated

4.3.1 Quick-spin the 96-Well Amplification plate(s) for approximately 30 seconds.

4.3.2 Within STaCS, select the daughter plate(s) to be processed and select the appropriate scenario.

4.3.2.1 Additionally, for automated processing only:

- Scan the instrument barcode on the Tecan EVO Robotic Workstation.
- Ensure the Robot Maintenance Checks have been performed.
- Indicate whether each check passed.

4.3.3 Scan the barcode on each of the 96-Well Amplification plate(s), the EPP Daughter plate(s) and each reagent required for the selected scenario.

4.3.4 Select "Process" and proceed with the EPP procedure.

4.3.4.1 For manual processing, remove the cover from the 96-Well Amplification plate.

4.3.4.2 Additionally, for automated processing only:

- STaCS launches the robotic software for the Tecan EVO Robotic Workstation
- If necessary, enter the appropriate user name and password at the robotic software log-in screen.

- Verify that the appropriate EPP script has been opened.
- Remove any plastic cover(s) on the 96-Well Amplification plate(s), if necessary.
- Ensure the selected 96-Well Amplification plate(s), the corresponding EPP Daughter plate(s) and the required reagents have been loaded on to the instrument.
 - Well A1 should be in the back right corner of the hotels.
- Ensure the instrument has been properly flushed and no air bubbles are visible in the tubing or syringes.
- Start the EPP script.
- Indicate the number of plates to be processed.

The following EPP procedure will be performed manually or by the Robotic Workstation:

- 4.3.5** Aliquot 24 µl of the GS-600 [LIZ] formamide solution into each well of the EPP Daughter plate(s).
- 4.3.6** Add 1 µl of PCR product from the 96-Well Amplification plate(s) to its corresponding sample well in the EPP Daughter plate(s) and 1 µl of the appropriate allelic ladder to the designated well(s) in the EPP Daughter plate(s).
- 4.3.7** The Tecan EVO Robotic Workstation will re-seal the 96-Well Amplification plate(s) with a plastic cover. For manually processed plate(s), heat seal the 96-Well Amplification plate(s) with a plastic cover.
- 4.3.8** Visually inspect the EPP Daughter Plate(s).
- 4.3.9** Indicate the result in STaCS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results. If the plate(s) were processed on the Tecan EVO Robotic Workstation, indicate in STaCS whether the bleach process was performed.
- 4.3.10** Ensure the EPP Daughter plate(s) are covered with septa.
- 4.3.11** Vortex (approximately 2 seconds) and quick-spin (approximately 30 seconds) the EPP Daughter Plate(s).
- 4.3.12** Return the 96-Well Amplification plate(s) to refrigerated storage in the post-amplification laboratory (4°C ± 3°C) until data analysis has been completed, at which time the plate(s) can be discarded.

4.4 Electrophoresis Preparation Denature

- 4.4.1** Load the EPP Daughter Plate(s) into the Thermal Cycler(s).
- 4.4.2** Slide the lid of the thermal cycler(s) forward over the EPP Daughter Plate(s). Do not close the lid of the thermal cycler during the denaturing step.

- 4.4.3** Select the appropriate method on the thermal cycler and verify that the method displayed on the instrument screen matches the method outlined below:

HOLD 95°C 3 minutes

HOLD 4°C 3 minutes

HOLD 4°C Forever

- 4.4.4** Start the thermal cycler. Verify that the reaction volume is 25 µl and the ramp speed is 9600.
- 4.4.5** Ensure the *Thermal Cycler Bar Code* and *EPP Daughter Plate Bar Code* for each plate to be denatured has been scanned into STaCS.
- 4.4.6** Select "Save" and indicate the result in STaCS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results.

4.5 Setting Up the Genetic Analyzer (Sequencer)

The type of AB Genetic Analyzer validated to perform capillary electrophoresis is based on the amplification kit and sample type as follows:

	Identifiler	Identifiler Direct
Blood	3130xl or 3730	N/A
Buccal	3130xl	3730

NOTE: *If sequencer general maintenance is required, refer to Appendix A for guidance.*

- 4.5.1** Start the computer attached to an appropriate AB Genetic Analyzer and log onto the workstation.
- 4.5.2** Ensure that the oven and all instrument doors are shut. Press the power button on the front of the analyzer to start the instrument. Ensure that the green status light is on before proceeding.
- 4.5.3** Launch the Data Collection Software and Service Console application. The Data Collection software will open once a green box is indicated for the *Messaging Service*, *Data Service*, *Instrument Service*, and *Viewer*. Expand the navigation panel on the left.

4.5.4 Replenish the buffer, water and waste reservoirs, if necessary.

	3130xl Genetic Analyzer	3730 Genetic Analyzer
Frequency (<i>whichever comes first</i>)	One week or 6 full 96-Well plates (~36 injections)	48 hours or 16 full 96-Well plates (~32 injections)
Anode Buffer Reservoir (1X Genetic Analyzer Buffer with EDTA)	Fill to the red line (~16mL) Place on the pump block	Fill to the red line (~67 mL) Place on the pump block
Cathode Buffer Reservoir (1X Genetic Analyzer Buffer with EDTA)	Fill reservoir to the fill line (~16mL) Place in position 1 on the autosampler	Fill reservoir to the line (~80 mL) Place in the left most position
Water and Waste reservoirs (water)	Fill three reservoirs to the fill line (~16mL each) Place in positions 2 through 4 on the autosampler	Fill two reservoirs to the line (~80 mL each) Place in middle and right positions, respectively

- To prepare 1X Genetic Analyzer Buffer, dilute the appropriate 10X Genetic Analyzer Buffer with water and record in the *Chemical Preparation* module in STaCS.
- Ensure cathode buffer, water, and waste reservoirs are covered with septa.

NOTE: To avoid electrical arcing, all surfaces of the reservoirs must be clean and dry.

4.5.5 The oven may be turned on and the temperature set in advance to shorten the interval between run activation and execution. It takes approximately 30 minutes to reach the target temperature.

From the *Manual Control* menu in the navigation panel:

3130xl Genetic Analyzer	3730 Genetic Analyzer
<ul style="list-style-type: none"> ○ <i>Send defined command for</i> = Oven ○ <i>Command Name</i> = Turn on/off oven ○ <i>Value</i> = On ○ Execute by selecting “Send Command” 	<ul style="list-style-type: none"> ○ <i>Send defined command for</i> = Oven ○ <i>Command Name</i> = Set oven state ○ <i>Value</i> = On ○ Execute by selecting “Send Command”
Set the oven to 60° C by selecting the following parameters: <ul style="list-style-type: none"> ○ <i>Send defined command for</i> = Oven ○ <i>Command Name</i> = Set oven temperature ○ <i>Value</i> = 60°C ○ Execute by selecting “Send Command” 	Set the oven to 66° C by selecting the following parameters: <ul style="list-style-type: none"> ○ <i>Send defined command for</i> = Oven ○ <i>Command Name</i> = Set oven temperature ○ <i>Value</i> = 66°C ○ Execute by selecting “Send Command”
	To turn the buffer heater on, select the following parameters: <ul style="list-style-type: none"> ○ <i>Send defined command for</i> = Autosampler ○ <i>Command Name</i> = Turn On/Off buffer heater ○ <i>Value</i> = On ○ Execute by selecting “Send Command”

NOTE: The oven and/or buffer reservoir status can be verified by selecting *Instrument Status* from the navigation panel.

- 4.5.6** Ensure the chemicals/reagents/array required for the Genetic Analyzer have been defined and/or verified in STaCS using the *Sequencer Configuration* module.
- 4.5.7** If necessary, ensure any changes to chemicals/reagents/array performed on the Genetic Analyzer have been recorded in STaCS using the *Instrument Maintenance* module.

4.6 Post PCR and Sample Sheet Creation

In the *Post PCR* module, the type of Genetic Analyzer (*Sequencer*) to be used for capillary electrophoresis is selected, the reagents assigned to the sequencer are recorded and a sample sheet(s) generated.

- 4.6.1** Within STaCS, scan the *Sequencer Bar Code* of the selected instrument that will be utilized to perform capillary electrophoresis.
- 4.6.2** Scan the barcode on the Electrophoresis (EPP) daughter plate(s) to be processed.
- 4.6.3** Specify the *Destination Directory* for the sample sheet(s) in STaCS.
- 4.6.4** Create the sample sheets. STaCS creates a sample sheet file with the same name as its corresponding EPP daughter plate barcode.

NOTE: If multiple injections of any FDDU samples and/or controls are required, the user may add the injections during the creation of the sample sheet or later in the *Data Collection* software of the sequencer. If extra injections are added during creation of the sample sheet, all samples and controls on the plate will have the additional injections included in the sample sheet.

- 4.6.5** Select "Save" and indicate the result in STaCS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results.

4.7 Initiating Capillary Electrophoresis on a 3130xl Genetic Analyzer

- 4.7.1** Import the STaCS generated sample sheets.
 - Choose *Plate Manager* from the navigation panel.
 - Select *Import* and browse to the STaCS generated sample sheet file(s).
 - Select *OK* to import the Plate Record(s) into the 3130xl Data Collection Software.
- 4.7.2** In the Plate Manager window, verify the following:
 - *Plate ID* and *Plate Name* = the corresponding STaCS daughter plate barcode
 - *Type* = GeneMapper
 - *Size* = 96

4.7.3 Review the Plate Record(s)

- In the *Plate Manager* window, highlight the plate and select *Edit* to open the *GeneMapper Plate Editor* spreadsheet
- Ensure the *Sample Name* column contains expected sample barcodes
- Ensure the *Sample Type* for Ladders and Controls and that the *Size Standard*, *Panel* and *Analysis Method* fields are complete
- Ensure the *Results Group* and *Instrument Protocol* fields are correct for the selected instrument
- Use the pull down menus to make any necessary changes to the spreadsheet

Note: The parameters (e.g., *Injection kV*) defined in an *Instrument Protocol/Panel* may vary between AB 3130xl Genetic Analyzers. The *Instrument Protocol/Panel(s)* for each instrument are available for reference.

4.7.4 If necessary, to add injections of any FDDU samples and/or controls not added during Post PCR and Sample Sheet Creation:

- Select “Add Sample Run” from the *Edit* menu to add additional *Results Group* and *Instrument Protocol* columns.
- Select the appropriate *Results Group* and *Instrument Protocol* parameters for the FDDU samples and/or controls that require additional injections.
- Select "OK" to save the Plate Record.

4.7.5 Ensure the AB 3130xl plate assemblies have been prepared with the EPP Daughter Plates and the plate assemblies are properly seated on the autosampler.

4.7.6 Select *Run Scheduler* from the navigation panel. In *Plate View*, the checkered box will change from gray to yellow when the plate assemblies are seated correctly. Link the EPP daughter plate(s) to their *Plate Record* (sample sheets).

- Select *Plate View* under the *Run Scheduler* on the navigation panel.
- Select the appropriate plate record for the EPP daughter plate in autosampler position A.
- Verify that the EPP daughter plate barcode in autosampler position A matches the selected *Plate Record*.
- Select the checkered area of the plate A position indicator.
- This activates the green *Start Run* icon located on the tool bar.
- Repeat, if necessary, to link the EPP daughter plate in position B.

4.7.7 Ensure that there are no bubbles in the tubing, array port or pump blocks. The Data Collection wizard for bubble removal may be run, if necessary. The capillary array may be filled with fresh polymer prior to starting the run.

- Choose *Manual Control* from the navigation panel
- *Send defined command for* = Polymer Delivery Pump
- *Command Name* = Fill 36 cm Capillary Array
- Execute by selecting “Send Command”

4.7.8 Ensure the doors on the AB 3130xl are closed and start the run.

- The Processing Plates dialog box opens and prompts the user that “You are about to start processing plates...”
- Select “OK”

Note: The electrophoresis run can be monitored by selecting *Instrument Status* from the navigation panel.

4.8 Initiating Capillary Electrophoresis on a 3730 DNA Analyzer

4.8.1 Import the STaCS generated sample sheets.

- Choose *Plate Manager* from the navigation panel.
- Select *Import* and browse to the STaCS generated sample sheet file(s).
- Select *Open* to import the Plate Record(s) into the 3730 Data Collection Software

4.8.2 In the Plate Manager window, verify the following:

- *Plate ID & Plate Name* = the corresponding STaCS daughter plate barcode
- *Type* = GeneMapper
- *Size*=96

4.8.3 Review the plate record for a minimum of one plate in each run:

- In the *Plate Manager* window, highlight the plate and select *Edit* to open the *GeneMapper Plate Editor* spreadsheet.
- Verify the *Sample Name* column contains expected sample barcodes.
- Verify the *Sample Type* for Ladders and Controls and that the *Size Standard*, *Panel* and *Analysis Method* fields are complete.
- Verify the *Results Group* and *Instrument Protocol* fields are correct for the selected instrument.
- Use the pull down menus to make any necessary changes to the spreadsheet.

NOTE: The parameters (e.g., Injection kV) defined in an *Instrument Protocol/Panel* may vary between AB 3730 DNA Analyzers. The *Instrument Protocol/Panel(s)* for each instrument are available for reference.

4.8.4 If necessary, to add injections of any FDDU samples and/or controls not added during Post PCR and Sample Sheet Creation:

- Select “Add Sample Run” from the *Edit* menu.
- Additional *Results Group* and *Instrument Protocol* columns are added to the *Genemapper Plate Editor* spreadsheet.
- Select the appropriate Results Group and Instrument Protocol parameters for only the FDDU samples and/or controls that require additional injections.
- Select "OK" to save the Plate Record.

4.8.5 Ensure the AB 3730 plate assemblies have been prepared with the EPP Daughter Plates and place the assemblies into the In Stacker tower on the plate stacker, which can hold up to 16 plates.

- 4.8.6** Verify that the EPP daughter plate barcode for each plate is clearly visible when the door to the In Stacker tower is open. This may require adjusting the barcode(s) to ensure they can be read by the internal barcode scanner.
- 4.8.7** Ensure the Out Stacker tower has sufficient space to process all plates to be run, close both tower doors and close the stacker drawer. This will home the autosampler causing the array to rest in the buffer reservoir.
- 4.8.8** Ensure the instrument Run Mode is on Auto:
- Select *Run Scheduler* from the navigation panel.
 - From the *Instrument* menu select *3730_# Run Mode*.
 - Verify that *Auto* is selected.
 - When *Auto* mode is selected and the EPP daughter plate assemblies are loaded into the plate stacker; the *Plate Id*, *Plate Name* and *Plate Type* columns in the Input Stack portion of the *Run Scheduler* window will read *Unknown*.
- 4.8.9** Ensure the Output Stack portion of the *Run Scheduler* window has sufficient space to process all plates to be run.
- 4.8.10** Ensure there are no bubbles in the tubing, array port or pump blocks. The Data Collection wizard for bubble removal may be run, if necessary.
- 4.8.11** Ensure the correct Dye Set and Spectral are set for the run by checking the *Dye Set* and *Active Calibration* in the *Spectral Viewer* window.
- 4.8.12** Start the Run.
- The Processing Plates dialog box opens and prompts the user that “You are about to start processing plates...”
 - Select “OK”.

NOTE: The electrophoresis run can be monitored by selecting *Instrument Status* from the navigation panel.

5 Sampling

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

The appropriate processing methods are selected for a plate based on the sample type added to the plate and the amplification kit to be used. Based on internal studies, only the combinations of processes listed below are approved for use.

Sample Type	Amp Kit	EPP	Genetic Analyzer
Blood	Identifiler	Manual or Automated using a Tecan EVO Robotic Workstation	AB 3130xl or AB 3730
Buccal	Identifiler	Manual only	AB 3130xl
	Identifiler Direct	Manual or Automated using a Tecan EVO Robotic Workstation	AB 3730

9 Safety

9.1 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.2 Procedural Specific Chemical Hazard:

- Formamide is a teratogen. Avoid inhalation, skin contact, or ingestion. Use nitrile gloves when handling. Dispose of unused portions in appropriate hazardous waste containers. Pregnant women must not handle formamide.
- Performance Optimized Polymer 4 is caustic. Avoid inhalation, skin contact, or ingestion. Use gloves when handling. Dispose of unused portions in appropriate hazardous waste containers.
- Performance Optimized Polymer 7 is caustic. Avoid inhalation, skin contact, or ingestion. Use gloves when handling. Dispose of unused portions in appropriate hazardous waste containers.

10 References

FBI Laboratory Quality Assurance Manual.

FBI Laboratory Safety Manual.

DNA Procedures Manual.

Federal Bureau of Investigation Quality Assurance Standards for DNA Databasing Laboratories, current version.

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Applied Biosystems. *AmpFISTR® Identifiler® PCR Amplification Kit Product Insert*, P/N 4322288, Applied Biosystems, Foster City, CA.

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Wang et al. Development and Validation of the AmpFISTR® Identifiler® Direct PCR Amplification Kit: Multiplex Assay for the Direct Amplification of Single-Source Samples, *Journal of Forensic Science* (2011) doi: 10.1111/j.1556-4029.2011.01757.x.

Rev #	Issue Date	History
7	06/26/14	Added clarification that control samples typed through capillary electrophoresis are included as applicable in section 4. Updated section number to reference in section 7.1.7 to reflect changes in numbering to this revision. Deleted need for instrument from sections 7.1.8 and 7.4.3.6. Deleted section 7.2.6 and associated footnotes and renumbered remainder of section. Deleted footnote 5. Deleted Appendix A and all associated references throughout document.
8	03/11/16	Revised entirety of document for simplification, clarity, and to remove computer interface requirements. Combined all FDDU post amplification laboratory procedures (306-7, 307-7, and 314-5). Relocated QA/QC guidance and procedures to Appendix A.

Approval

Redacted - Signatures on File

Appendix A: CE Maintenance and Performance Verification Procedures

General maintenance and performance verification (PV) procedures are performed in accordance with the LOM practice and DNA procedure for equipment calibration and maintenance. General maintenance is generally performed at the intervals listed below. Performance verification of the genetic analyzer instruments will be conducted at the minimum frequency described in the DNA procedure for equipment calibration and maintenance.

General Maintenance	Recommended Interval	
	3130 xl	3730
Replace Polymer	Weekly	Weekly
Replace 1x Buffer/Water/Waste	Weekly (or as required in procedure)	Weekly (or as required in procedure)
Water Wash	Weekly	Weekly
Flush Water Trap	Weekly	Weekly
Database Cleanup	Monthly	Weekly
Data Backup	Monthly	Weekly
Disk Defragmentation	Monthly	Monthly
Install New Array	As needed	As needed
Spatial Calibration	With array change or as needed	Weekly
Spectral Calibration	As needed	Weekly
Sensitivity Evaluation	After optical adjustment	Quarterly or after optical adjustment

I. General Maintenance of the Applied Biosystems 3130xl

Weekly

1. Preparing new bottle of POP-4
 - a. Loosen the POP-4 bottle cap and allow it to sit on the bench top for approximately 15 minutes to degas.
2. Flush the polymer delivery pump (PDP)
 - a. Run the "water wash wizard" and use reagent grade water to flush the PDP.
Note: For a warm water wash, heat water to < 60 °C.
 - b. Follow wizard prompts.
3. Flush the PDP water trap
 - a. Use a 20 mL Luer lock syringe filled with reagent grade water.
 - b. Attach the syringe to the forward facing Luer fitting at the top of the pump block, open the Luer approximately one-half turn counter clockwise.
 - c. Open the exit fitting at the top left side of the pump block approximately one-half turn counter clockwise.
 - d. Flush the water trap with approximately 5 mL of water
 - e. Close both fittings by turning them clock wise until finger-tight, do not over tighten.

Monthly

1. Maintenance of the storage databases used by the Data Collection software.
 - a. Open the appropriate results group folder and create a new backup folder using the naming convention, CE #XX_Backup_MMDDYY.
 - b. Move all plate folders into the newly created backup folder and then copy it to the appropriate CE Backup folder on the network. (Example: \\FS1\CE Backup)
2. Delete records from the database
 - a. From the navigation pane, select "Database Manager" and "Cleanup Processed Plates".
 - b. Allow the software the appropriate amount of time to delete the associated records and close the dialog box once complete.
3. Defragment the data storage hard drive using the disk defragmenter in Windows system tools to defragment the (E:) drive.

II. General Maintenance of the Applied Biosystems 3730

Weekly

1. Preparing new bottle of POP-7
 - a. Loosen the POP-7 bottle cap and allow it to sit on the bench top for approximately 15 minutes to degas.
2. Flush the polymer delivery pump (PDP)
 - a. Run the "water wash wizard" and use reagent grade water to flush the PDP.
Note: For a warm water wash, heat water to $< 60^{\circ}\text{C}$.
 - b. Follow wizard prompts.
3. Flush the PDP water trap
 - a. Use a 20 mL Luer lock syringe filled with reagent grade water.
 - b. Attach the syringe to the forward facing Luer fitting at the top of the pump block, open the Luer approximately one-half turn counter clockwise.
 - c. Open the exit fitting at the top left side of the pump block approximately one-half turn counter clockwise.
 - d. Flush the water trap with approximately 5 mL of water
 - e. Close both fittings by turning them clock wise until finger-tight, do not over tighten.
4. Perform a spatial calibration upon completion of the "water wash wizard".
 - a. Refer to spatial calibration procedure at the end of the appendix for instructions and pass/fail criteria.
5. Perform a spectral calibration following successful spatial calibration.
 - a. Refer to spectral calibration procedure at the end of the appendix for instructions and pass/fail criteria.
6. Maintenance of the storage databases used by the Data Collection software.
 - a. Open the appropriate results group folder and create a new backup folder using the naming convention, CE#XX_Backup_MMDDYY.
 - b. Move all plate folders into the newly created backup folder and then copy it to the appropriate CE Backup folder on the network. (Example: \\FS1\CE Backup)
7. Delete records from the database
 - a. From the navigation pane, select "Database Manager" and "Cleanup Processed Plates".

- b. Allow the software the appropriate amount of time to delete the associated records and close the dialog box once complete.

Monthly

1. Defragment the data storage hard drive using the disk defragmenter in Windows system tools to defragment the (E:) drive.

III. Array Change and Spatial Calibration

The capillary array will be changed as needed. The determination to change the array will be based upon a review of the quality of the data generated by the instrument. Generally, the array on the 3130xl should be changed after 150 injections.

1. From the toolbar select the “Install Array Wizard”.
2. Install the array as instructed by the wizard.
 - a. Ensure the proper type (16 or 48) and length (36 cm) is entered in the array information fields.
3. In the final step of the wizard you can choose to fill the array with polymer or click “finish” if the array will be filled during the spatial calibration.

A spatial calibration must be performed whenever a new array is installed, every time the detection cell window is opened, or each week for a 3730.

1. Select “Spatial Run Scheduler” in the navigation pane.
2. Select “SpatialFill_1”
3. Click the “Start” button to initiate the spatial calibration.

Note: “SpatialNoFill_1” can be selected if there is no need to fill the array with fresh polymer.
4. Select “Accept” to accept the spatial calibration if the following criteria are met:
 - a. Peaks of the spatial calibration are approximately the same height.
 - b. An orange cross appears at the top (apex) of each peak in the profile.
 - c. No irregular peaks are contained in the profile
 - d. RFU values for the peaks are greater than 2,000 for a 3130 array and 1,000 for a 3730 array
 - e. The values for the Left Spacing and Right Spacing columns for a 3130 array are 13-16 pixels and 9-11 pixels for a 3730 array. (A spatial calibration can be accepted if one or more of the spacing values lie outside of this range but it is preferable to have all the values within this specification.)

IV. Spectral Calibration

A spectral calibration is generally run each quarter for a 3130 and weekly for a 3730. While not necessary, a spectral calibration is recommended after changing the capillary array. A spectral plate may be injected several times within a 24 hour period, a fresh spectral plate should be used for each instrument being calibrated.

3130xl	3730
<ol style="list-style-type: none"> 1. Combine 195 uL of formamide with 5 uL of DS-33 Matrix Standard 2. Dispense 10 uL of solution into wells A1-H1 and wells A2-H2. 3. Spin down and denature plate on thermal cycler then place on instrument. 4. Click "Plate Manager" in the navigation pane.* 5. Select "New" and the "New Plate Dialog" dialog box will open, fill out fields as follows <ol style="list-style-type: none"> a. Name: Use the naming convention CE#XX_Spectral_MMDDYY. b. Select "Spectral Calibration" from the "Application" drop down menu. c. Complete the remaining fields and select "OK". This will open the "Spectral Calibration Plate Editor" window. 6. Create sample sheet. <ol style="list-style-type: none"> a. Fill out the "Sample Name" fields to mirror the plate layout. b. Select "Spectral_G5" from the drop down in the "Instrument Protocol 1" field. c. Press "OK" to save plate sample sheet. 7. Select "Run Scheduler" from the navigation pane <ol style="list-style-type: none"> a. Search the plate name or select find all and click on the plate to be run in order to highlight it within the list. b. Click "Link" to associate the sample sheet to the plates location on the instrument. 8. Click the green arrow to start processing the spectral plate. 	<ol style="list-style-type: none"> 1. Combine 987 uL of formamide with 13 uL of DS-33 Matrix Standard 2. Dispense 10 uL of solution into wells A1-H1, A3-H3, A5-H5, A7-H7, A9-H9, and A11-H11 3. Spin down and denature plate on thermal cycler then place on instrument. 4. Click "Plate Manager" in the navigation pane.* 5. Select "New" and the "New Plate Dialog" dialog box will open, fill out fields as follows <ol style="list-style-type: none"> a. Name: Use the naming convention CE#XX_Spectral_MMDDYY. i. enter the same information in the "ID (Barcode)" field if running in manual mode, otherwise enter the plates barcode ID in this field. b. Select "Spectral Calibration" from the "Application" drop down menu. c. Select "Septa" from the "Plate Sealing" drop down menu d. Complete the remaining fields and select "OK". This will open the "Spectral Calibration Plate Editor" window. 6. Create sample sheet. <ol style="list-style-type: none"> a. Fill out the "Sample Name" fields to mirror the plate layout. b. Select "Spectral_G5-RCT" from the drop down in the "Instrument Protocol 1" field. c. Press "OK" to save plate sample sheet. 7. Select "Run Scheduler" from the navigation pane <ol style="list-style-type: none"> a. Search the plate name or select find all and click on the plate to be run in order to highlight it within the list. b. Click "Add" to add the plate to the input stack. 8. Click the green arrow to start processing the spectral plate.

* A previously created spectral plate can be duplicated by highlighting a plate in the Plate Manager window and clicking "Duplicate". This method will only require a new plate name be entered while retaining all the previously entered information.

Pass Criteria: The data collection software indicates the pass/fail status of each capillary. The spectral calibration is acceptable if the following criteria are met, and there is proper separation between the color channels.

Instrument	Number of failing wells	Minimum Peak height
3130xl	≤ 3caps, no more than 2 in a row	2,000 RFU
3730	≤ 5 caps, no more than 2 in a row	500 RFU

The Data Collection software automatically applies a passing spectral and no further action is required by the user.

V. Performance Verification and Sensitivity Evaluation

Performance verification of the genetic analyzer instruments should be conducted at the minimum frequency described in the DNA procedure for equipment calibration and maintenance. A Sensitivity Evaluation may be run in lieu of a Performance Verification and must be run after any optical adjustment is made to the instrument.

3130xl	3730
<ol style="list-style-type: none"> 1. Amplify 9947A (Diluted/Promega) with the Identifiler amplification kit for 24 cycles. 2. Prepare a stock solution in a 24:1 ratio of GS600LIZ/Formamide to amplicon. 3. Add 25 uL of stock solution to wells A1-G1 and A2-G2, 4. Add 1 uL of Identifiler ladder and 24 uL of GS600LIZ/Formamide solution to wells H1 and H2. 5. Inject the samples three times at the instruments current injection setting. 	<ol style="list-style-type: none"> 1. Amplify 9947A (Diluted/Promega) with the Identifiler amplification kit for 24 cycles. 2. Prepare a stock solution in a 24:1 ratio of GS600LIZ/Formamide to amplicon. 3. Add 25 uL of stock solution to wells A1-G1, A3-H3, A5-G5, A7-H7, A9-H9, A11-G11. 4. Add 1 uL of Identifiler ladder and 24 uL of GS600LIZ/Formamide solution to wells H1, H5, and H11. 5. Inject the samples two times at the instruments current injection setting
Additionally for Sensitivity Evaluation:	
<ol style="list-style-type: none"> 6. Inject the samples three times, at various voltage settings above and/or below the previously determined injection voltage. 	<ol style="list-style-type: none"> 6. Inject the samples two times, at various voltage settings above and/or below the previously determined injection voltage.

Pass Criteria: A Genetic Analyzer will be deemed suitable for FDDU analysis if:

1. Average peak heights of all alleles in the 9947A positive control are greater than 150 RFU and
2. Correct typing results obtained for 9947.

Injection voltage may be adjusted based on the evaluation of the data generated at the various injection voltage settings.

FDDU

Procedures for CODIS Entry and Uploading of Database STR Profiles

1 Scope

These procedures apply to the entry and uploading of database profiles into **Redacted** of the **COmbined DNA Index System (CODIS)**. CODIS functions as three separate tiers: the Local DNA Index System (LDIS), the State DNA Index System (SDIS) and the National DNA Index System (NDIS).

2 Equipment/Materials/Reagents

STACS (Sample Tracking and Control System) Software (STACS DNA Inc.), version 6.0 or higher.

CODIS (Combined DNA Index System) Software (FBI Laboratory), version 7.0 or higher

3 Procedures

3.1 CODIS Specimen ID and Specimen Categories

3.1.1 DNA profiles from database samples are entered into CODIS using a standardized format. Generally, the Specimen ID is the unique STACS Sample ID number for each sample (e.g., **Redacted**

3.1.2 DNA profiles from database samples may be entered into one of the following Specimen Categories/CODIS Indexes:

- Convicted Offender
- Arrestee
- Detainee

3.1.2.1 The Specimen Category/CODIS Index for each DNA profile is generally determined based on the *Contributor Type* selected in STACS for each sample

3.2 Data Review Requirements Prior to CODIS Entry and Uploading

3.2.1 The genetic analysis data for all short tandem repeat (STR) database profiles to be entered into CODIS must undergo primary analysis and technical (secondary) review by an STR qualified examiner. Interpretation/review of the data must be performed in accordance with the appropriate interpretation protocol (i.e., FDDU 315).

3.2.2 For all database samples, analysis of all applicable CODIS Core Loci must be attempted for CODIS entry and NDIS registration.

3.2.3 Each qualified Examiner and CODIS Biologist (Technical Specialist) must complete the "Annual NDIS Eligibility Training" which is required per the NDIS procedures.

3.3 Entering and Uploading STR Profiles into CODIS

Profiles submitted to LDIS, SDIS and NDIS will be entered into the appropriate specimen category (e.g., Convicted Offender, Juvenile, Multi-allelic Offender, Arrestee, Detainee) and into the appropriate index (e.g., Offender, Multi-allelic Offender, Arrestee, Detainee).

3.3.1 A CMF file containing STR profiles is entered into LDIS by an active **Redacted** user. Data entry and uploading of STR profiles will be performed following the NDIS procedures and instructions for the current version of the CODIS software.

3.3.2 If necessary, STR profiles may be manually entered into or edited in LDIS using the CODIS STR/Y-STR Data Entry in the Specimen Manager module. Profiles that are manually entered into LDIS must be verified by the CODIS Program Manager (or designee).

3.3.3 The STR profiles at LDIS are marked for upload to SDIS according to parameters set by the FBI CODIS State Administrator (or designee).

Redacted

3.3.4 The STR profiles at SDIS are marked for upload to NDIS according to parameters set by the NDIS Custodian.

3.3.4.1 An STR profile developed from a sample entered into **Redacted** (Convicted Offender, Multi-allelic Offender, Arrestee or Detainee) must include all appropriate CODIS core for NDIS registration and searching.

3.3.5 An active **Redacted** user (typically an FDDU Examiner or CODIS Biologist) must review the SDIS and NDIS Reconciliation Reports and confirm or reject samples in the STACS *CODIS Confirmation* module. The decision to confirm or reject a sample is based on whether the sample was successfully uploaded to CODIS.

3.4 Routine Searches of the Database

3.4.1 Autosearches of the FBI SDIS are conducted based on the parameters set by the FBI CODIS State Administrator (or designee). Autosearches of NDIS are conducted based on the parameters set by the FBI NDIS Custodian (or designee).

Redacted

Redacted

3.4.3 An active Redacted user will routinely check the CODIS software for candidate matches.

3.5 Confirming and Releasing Matches

3.5.1 Until the confirmation of the match is ready for release, information regarding the match will be provided on a need-to-know basis.

3.5.2 Matches will be verified and communicated by an STR qualified Examiner as outlined in the appropriate DNA procedure (i.e., FDDU 311).

3.5.3 If it is determined that duplicate database samples have been entered into CODIS, the duplicate sample will be unmarked in CODIS.

3.6 CODIS Search Requests

3.6.1 All STR Batch Target File search requests are currently submitted via the FBI CODIS Unit and made available to all CODIS laboratories through the CODIS web page. The STR Batch Target Files are available for download. An active Redacted user will update the target batch file and search against database samples at a minimum of once a month.

3.6.2 A candidate match found with a target profile will be provided to the Casework Laboratory for review and evaluation. Upon notification from the Casework Laboratory, the FDDU will confirm the match following the appropriate DNA procedure (i.e., FDDU 311).

3.6.3 One-time search requests of LDIS/SDIS for DNA profiles from other NDIS participating laboratories will be individually evaluated by the LDIS and/or FBI SDIS Administrators (or designee) prior to initiating a manual keyboard search.

3.6.4 No “victim” or “suspect” searches will be conducted of the FBI LDIS/SDIS database STR profiles.

Redacted

Redacted

4 Standards and Controls

Not applicable.

5 Sampling

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

Not applicable.

9 Safety

Not applicable.

10 References

DNA Procedures Manual

FBI Laboratory Quality Assurance Manual

National DNA Index System (NDIS) Operational Procedures Manual, current version

STaCS DNA Inc. *Sample Tracking and Control System (STaCS) User's Guide*.

Quality Assurance Standards for DNA Databasing Laboratories, current version.

Rev. #	Issue Date	History
3	05/01/13	Made changes throughout entire document to reflect upgrade in CODIS software in November 2011 from CODIS 5.7.4 to CODIS 7.0. Broadened language to include all missing persons-related samples, and added language to clarify what type of convicted individuals are entered into LDIS in Section 2. Deleted Sections 7.1 through 7.3 and renumbered remainder of document. Updated description of Sample ID in Section 7.1.1 and footnote 1. Added data review requirements for outsourced STR profiles in Section 7.1.2.2. Updated requirement to attempt analysis of all 13 of CODIS core loci and upload requirement of 10 for SDIS and 13 for NDIS in Sections 7.1.2.3, 7.1.6.3 and 7.1.6.5. Deleted Profiler Plus and COfiler from Section 7.1.4.2. Added language to clarify footnote 5. Updated definition of CMF files in 7.1.5. Added instructions on filtering samples for CODIS upload worklist to Section 7.1.5.2. Added language to clarify Section 7.1.6. Changed procedure in Sections 7.1.6.3 and 7.1.6.6. Edited wording for clarity in Sections 7.1.7.8 and 7.1.8.2. Added more detail to Routine Searches to Section 7.1.8 and renumbered remainder of section. Added Section 7.1.9.3 to address duplicate samples. Updated Section 7.1.10 (CODIS Search Requests). Deleted Section 7.4.11 that details Manual Keyboard Searches by NDIS Custodian and renumbered remainder of section. Updated Section 7.1.11. Deleted Section 7.4.12.2. Updated references in Section 12. Updated STaCS screen shots throughout entire document. Edited grammar and punctuation throughout entire document. Deleted Appendices A, B, and C.
4	12/09/16	Simplified entirety of document and made changes to reflect the expansion of the CODIS Core Loci.

Approval

Redacted - Signatures on File

FDDU Procedures for the Expungement and/or Removal of a Sample

1 Scope

These procedures apply to Federal DNA Database Unit (FDDU) personnel who determine whether an expungement or removal is required and who remove the DNA profile and destroy the DNA sample in response to a proper request for expungement/removal.

2 Background

The *DNA Fingerprint Act of 2005* (42 U.S.C. 13701) amended section 210304 of the *DNA Identification Act of 1994* (42 U.S.C 14132) to include the following:

The Director of the Federal Bureau of Investigation shall promptly expunge from the index described in subsection (a) the DNA analysis of a person included in the index –

- (i) on the basis of conviction for a qualifying Federal offense¹ or a qualifying District of Columbia offense (as determined under sections 3 and 4 of the *DNA Backlog Elimination Act of 2000* (42 U.S.C 14135a, 14135b),² respectively, if the Director receives, for each conviction of the person of a qualifying offense, a certified copy of a final court order establishing that such conviction has been overturned; or
- (ii) on the basis of an arrest under the authority of the United States, if the Attorney General receives, for each charge against the person on the basis of which analysis was or could have been included in the index, a certified copy of a final court order³ establishing that such charge has been dismissed or has resulted in an acquittal or that no charge was filed within the applicable time period.

There are three categories of removals: Legal Expungement, Administrative Removal, and Quantity Not Sufficient (QNS) Removal.

Legal: A Legal Expungement is a complete removal of a DNA profile from Sample Tracking and Control Software (STACS) and COmbined DNA Index System (CODIS) due to an overturned conviction, or for arrests, if charges have been dismissed or have resulted in an acquittal or that no charge(s) was filed. The sample(s) submitted to the FDDU (e.g., liquid blood, bloodstain cards, buccal cards) and any extracted/amplified DNA (if applicable) must be

¹ A qualifying Federal offense is defined in section 3(d) of the DNA Analysis Backlog Elimination Act of 2000; section 503 of the USA Patriot Act of 2001; section 203(b) of the Justice For All Act of 2004; section 1004(a) of the DNA Fingerprint Act of 2005; section 155 of the Adam Walsh Child Protection and Safety Act of 2006.

² For a qualifying District of Columbia offense, also refer to District of Columbia Act 14-77.

³ A court order is not considered "final" if time remains for an appeal or application for discretionary review with respect to the order.

destroyed. Also, the information obtained from the FD-936 *Request for National DNA Database Entry* must be deleted from STACS.

Administrative Removal: An Administrative Removal is generally warranted under one or more of the following circumstances:

- It is determined that the DNA collection was not pursuant to the current Federal legislation which requires that DNA samples are collected by agencies of the United States from individuals who are arrested, facing charges, or convicted (Federal, Military, or District of Columbia offense), and from non-U.S. persons who are detained under the authority of the United States, or
- The collection agency or other appropriate authority provides written notification that such a removal is warranted, or
- A procedural issue arises regarding the validity of a sample.

Quantity Not Sufficient (QNS) Removal: A QNS Removal is performed when a sample fails to yield a successful DNA profile following exhaustive testing or a sample is determined to be inadequate for DNA testing. A QNS Removal must be performed in order to remove the sample from the processing work lists in STACS.

3 Equipment/ Materials/ Reagents

CODIS Software (FBI Laboratory)

Laserfiche Software (Laserfiche Inc.)

Sample Tracking and Control System (STACS) Software (STACS DNA Inc.)

4 Procedures

4.1 Logging a New Expungement/Removal into STACS

4.1.1 All requests for expungement/removal must be logged into the Sample Expungement module of STACS, if a sample has been received by the FDDU.

4.1.2 Samples can be removed from processing in STACS and made available for expungement/removal at any point in the analytical process. Alternatively, a sample may be designated for expungement/removal by creating an alert for the submission in the STACS *BOLO* module (see FDDU 302).

4.1.3 A new sample expungement record is initiated in the Sample Expungement module of STACS. The user must ensure that the following items are appropriately selected:

- Type of expungement/removal: Legal, Admin (Administrative Removal), or QNS.

- Recipient of expungement/removal correspondence
- Subject of expungement/removal
 - If no submission is found, notify the individual requesting the expungement/removal that a sample has not been received by the FDDU (see Appendix A for example language and format).
- Status of expungement/removal
 - **Pending:** A status of *Pending* is the default status that is given to all expungement/removal requests, and indicates that the sample is in the process of being expunged/removed.
 - **Confirmed:** A status of *Confirmed* is given to an expungement/removal that has been confirmed as a valid request and the appropriate steps have been completed. See Section 4.5.
 - **Invalid:** A status of *Invalid* is given to an expungement/removal request that is determined to be invalid (e.g., invalid request for removal, additional qualifying offenses). See Section 4.4.

4.2 Legal Expungement

4.2.1 Requests for Legal Expungements must be submitted as a written or electronic request. Requests relating to expungement of qualifying state or Department of Defense convictions or arrests shall be referred to the appropriate state or the Department of Defense.

4.2.2 A request for a Legal Expungement must be received from the person seeking the expungement or his/her legal representative. A valid request for a Legal Expungement must include sufficient identifying information **Redacted** to determine if the person is included in the FDDU DNA database.

4.2.3 The request for a Legal Expungement must be accompanied by a certified copy of the final court order dismissing the arrest or conviction of the subject. The copy of the final court order must contain a certification that it is a true and accurate copy of the original court order and be signed and dated by an appropriate court official such as a court clerk. The court order itself shall be signed by a judge, dated, and include sufficient identifying information **Redacted** to determine the identity of the person.

4.2.4 If the request for a Legal Expungement does not include a copy of the final court order, the sender may be contacted to provide additional records. If additional records cannot be provided, the requestor will be notified in writing as to the reason the Legal Expungement cannot be processed. Change the status of the Legal Expungement to *Invalid*, see Section 4.4.

4.3 Administrative Removal

4.3.1 FDDU personnel are responsible for ensuring the accuracy and integrity of the DNA data in the FDDU DNA database.

4.3.2 In the event of an administrative error during sample collection (e.g., collection of a DNA sample from an individual without a qualifying status), written or electronic notification may be provided to the FDDU stating the subject involved and why the sample should not be included in the FDDU DNA database. If necessary, FDDU will contact the submitting agency or other appropriate authority to clarify or confirm the reason why the sample should not have been submitted. In addition, proper identifying information **Redacted** must be provided in order to identify the offender sample in question.

When it is determined through other FDDU processes (see FDDU 302 and 311) that a sample was collected from the incorrect person, the administrative removal of the sample can be initiated by FDDU personnel. Records must be available to justify the administrative removal.

4.3.3 If FDDU personnel determine that the DNA sample cannot be included in the FDDU DNA database, the Administrative Removal process will continue. If it is determined that the request is invalid, the requestor may be contacted to provide additional supporting records. If additional records cannot be provided, the requestor will be notified in writing as to the reason for such determination and the status of the Administrative Removal will be changed to *Invalid*, see Section 4.4.

4.4 Invalid Requests for Legal Expungements/Administrative Removals

4.4.1 The reason for the invalid status must be recorded in the expungement record. Change the status of the expungement record from *Pending* to *Invalid*.

4.4.2 A *Rejection Letter* detailing why the sample cannot be expunged/removed must be generated and sent to the requestor.

4.5 Valid Requests for Legal Expungements and Administrative Removals

4.5.1 Complete and record all applicable items on the *Expungement/Removal Checklist* in STACS as follows:

- a. Required for Legal Expungements only: Review the criminal history of the subject to corroborate that the person was included in the FDDU DNA Database for an offense that was overturned or the charges were dismissed pursuant to court order, and that the individual has no other qualifying status.

Redacted

- c. Retrieve the sample(s) from the appropriate storage location. With a witness present, destroy the sample(s) by placing in the appropriate biohazard trash receptacle. Both individuals must record the destruction of the sample
 - i. If any extracted and/or amplified DNA exists, it must also be destroyed.
- d. Retrieve the FD-936 (if available) and mark "EXPUNGED" at the top of the form.

Redacted

- g. Electronic copies of supporting records will be maintained in STACS and/or Laserfiche.

4.5.2 The *Confirmation Letter* recording the completion of the expungement/removal process must be generated and sent to the appropriate person (e.g., collector, requestor).

NOTE: The subject information in STACS must not be deleted until the *Confirmation Letter* has been generated.

4.5.3 Once the checklist has been properly completed in STACS and the *Confirmation Letter* has been generated, change the status of the expungement record from *Pending* to *Confirmed*.

4.5.4 If not automatically performed by the STACS software, all identifying information and the FD-936 image must be deleted from the submission record.

4.6 Quantity Not Sufficient (QNS) Removal

4.6.1 In the Sample Expungement module, open appropriate sample record on the work list, and select/verify the individual/agency to receive notification.

4.6.2 Change the status from *Pending* to *Confirmed*. A *Re-submission Letter* must be generated and sent to the collector or other appropriate person.

4.7 Special Case: Expunging/Removing a Matched DNA Profile

If it is determined that a DNA profile and the data relating to a candidate match must be expunged/removed, FDDU personnel must notify the other laboratory in writing that an expungement/removal is being performed. A copy of this notification will be maintained with the expungement record. Proceed with appropriate expungement and/or removal procedures (see Section 4.5).

4.7.1 A copy of the records requesting expungement or removal (i.e., verification of no qualifying offense), the *Expungement/Removal Checklist* and the expungement letter should be maintained with the appropriate Match Confirmation File.

4.7.2 When a valid Legal Expungement request is received and a Match Confirmation Letter has already been issued, the other laboratory(ies) involved in the candidate match(es) will be notified by the FDDU that an expungement is being performed. In addition, the NDIS Custodian must be contacted (e.g., via e-mail, phone) to remove all candidate matches associated with the DNA record. The State CODIS Administrator may also be notified.

5 Limitations

5.1 A legal expungement cannot be performed without a certified court order signed by a Judge.

5.2 An administrative removal cannot be performed without written or electronic records stating the reason for removal.

6 Safety

6.1 All FDDU samples are considered potentially infectious regardless of the perceived status of the source individual or the age of the material. All FDDU personnel who work with such material will follow the “Bloodborne Pathogen Exposure Control Plan” found in the most current version of the *FBI Laboratory Safety Manual*.

6.2 Refer to the “Safe Work Practices and Procedures”, “Bloodborne Pathogen Exposure Control Plan”, “Personal Protective Equipment”, and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual* for important personal safety information prior to conducting these procedures.

6.3 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

7 References

Federal Bureau of Investigation. Quality Assurance Standards for DNA Databasing Laboratories, current version.

FDDU Quality Assurance Manual

FBI Laboratory Quality Assurance Manual

FBI Laboratory Safety Manual

National DNA Index System Procedures

STACS DNA Inc. *Sample Tracking and Control System (STACS) User's Guide*.

DNA Identification Act of 1994 (42 U.S.C. 14132)

DNA Backlog Elimination Act of 2000 (42 U.S.C. 14135, P.L. 106-546, H.R. 4640)

USA Patriot Act of 2001 (P.L. 107-56, H.R. 3162)

Justice for All Act of 2004 (P.L. 108-405, H.R. 5107)

DNA Fingerprint Act of 2005 (P.L. 109-162, H.R. 3402)

Adam Walsh Child Protection and Safety Act of 2006 (42 U.S.C. 16901, P.L. 109-248, H.R. 4472)

District of Columbia Act 14-77. *DNA Sample Collection Emergency Act of 2001*. [Bill 14-231], Title 48 District of Columbia Regulations (DCR) Section 5938

District of Columbia DC Code 22-4151

Title 28 Code of Federal Regulations (CFR), Section 28.2

Rev. #	Issue Date	History
3	06/26/2014	Clarified Administrative Removals in section 2 so that one or more of the circumstances are needed. In section 7.4.3.2, deleted paragraph regarding procedure for when a notification is received that a sample should not be included in the database. Updated procedure for administrative removals in section 7.4.3.2. Added examples of biographical information to section 7.4.5.5(g). Updated procedure for QNS removals in section 7.4.6.1 Deleted section 10.2. Edited section 11.1 so that all FDDU samples are considered potentially infectious. Deleted Appendices A-D and all associated references throughout entire document, and renamed Appendix E as Appendix A to reflect this change.
4	05/03/2017	Streamlined and updated document to reflect changes due to implementation of electronic checklist and upgraded expungement module in STACS. Removed FDDU Chief or designee as appropriate FDDU personnel can perform most tasks. Changed document to record throughout.
5	01/15/2020	Updated the scope to apply to personnel. Updated equipment list 4.2.2 Removed and qualifying offense Reworded 4.2.4 Edited section 4.5.1 to update for destruction of FD-936 prior to request or palletization for large-scale destruction, and removal from Laserfiche. 4.7 Simplified to other laboratory 4.7.1 Revised to Match Confirmation File

Approval

Redacted - Signatures on File

FDDU Chief

Date: 01/14/2020

DNA Technical Leader

Date: 01/14/2020

Appendix A: *Example Letter for Sample Removal Request - No Sample Received*

Redacted - Form on File

FDDU Procedures for Data Analysis and Interpretation of DNA Samples using GeneMapper® ID-X Expert System Software

1 Scope

These procedures apply to DNA personnel who perform data analysis, interpretation, and technical review of the DNA typing results from FDDU samples using the Applied BioSystems (AB) GeneMapper® ID-X (GMIDX) Expert System Software as well as the import of the DNA profiles into the Sample Tracking and Control System (STACS) software. These procedures apply to the interpretation of samples amplified using either the AmpF/STR® Identifiler® (ID), Identifiler® Direct (IDD) or GlobalFiler® Express (GFE) Amplification Kit and analyzed on an AB 3130xl Genetic Analyzer, an AB 3730 DNA Analyzer, or an AB 3500xL Genetic Analyzer.

2 Equipment/Materials/Reagents

STACS Software (STACS DNA Inc.), version 6.0 or higher

GeneMapper® ID-X Software (Applied BioSystems), version 1.3 or higher (For ID and IDD) or version 1.4 or higher (For GFE)

3 Standards and Controls

See the Interpretation Guidelines Section of this document for the Evaluation of Internal Size Standards, Allelic Ladders and Control Samples.

4 Quality Control Procedures

4.1 Random Reanalysis of FDDU DNA Samples

FDDU samples reanalyzed during the year as part of the FDDU Procedures for CODIS Candidate Match Confirmation demonstrate random reanalysis.

4.2 GeneMapper® ID-X Quarterly Performance Check

Each quarter, the GMIDX Software must undergo a performance check. A minimum of 200 FDDU samples and corresponding quality controls will be processed through the software as detailed in these standard operating procedures on each approved amplification kit/platform combination. All samples and controls, both acceptable and unacceptable, will be evaluated to ensure that the software is still performing at optimum levels. If there are any samples that were deemed acceptable that should have been flagged for manual review, the incident will be recorded and reviewed further to find the source of the inconsistency. If it is determined that the expert system made a mistake (i.e., made an incorrect allele call), the expert system functionality will not

be used to review FDDU sample data until the cause of the mistake is determined and the appropriate expert system settings adjusted as necessary. All records will be maintained.

5 Post PCR Review

The STACS *Post PCR Review* module provides an optional mechanism for the preliminary review of genetic analysis data generated by the AB 3130xl, AB 3730 or AB 3500xL. The preliminary review may be limited to a general evaluation of the samples/controls and the overall quality of the plate (e.g., Loss of Resolution [LOR]) or it may include a more detailed review resulting in sample(s) being sent back for rework.

5.1 Within STACS, select the plate to be reviewed.

5.2 Analyzing a GeneMapper® ID-X Project

5.2.1 Open the GMIDX Software if not already open, browse to the folder containing the sample files to be analyzed and add samples.

5.2.2 Ensure the correct *Table Setting* is selected.

5.2.3 The following field settings are available for use based on the instrument and kit:

Amp Kit	Instrument	Analysis Method	Panel	Size Standard
Identifiler	3130xl	FBI GMIDX 3130XL ID (GS600)	Identifiler_v1.1x	GS600 LIZ
		FBI GMIDX 3130XL ID (GS600) 50 RFU		
	3730	FBI GMIDX 3730 ID	Identifiler_v1.2x	
		FBI GMIDX 3730 ID 50 RFU		
Identifiler Direct	3730	FBI GMIDX 3730 ID Direct	Identifiler_Direct _v1.2x	
		FBI GMIDX 3730 ID Direct 50 RFU		
Globalfiler Express	3500xL	FBI GMIDX 3500 GFE	Globalfiler_Express _v1.2x	GS600_LIZ+ Normalization _(60-460)
		FBI GMIDX 3500 GFE (75RFU)		

5.2.4 Evaluate the electronic data in GMIDX using the *Raw Data* tab for each Negative or Combo control and Amplification Blank, as applicable, to verify the presence of a primer peak in

the raw data.

5.2.5 The analysis method settings can be viewed and, if needed, the range(s) adjusted by selecting *Analysis Method Editor* (icon with a small broad blue peak) from the tool bar on the top of the screen. The analysis settings for each kit and instrument combination are maintained with the validation and/or performance check records. Normalization is applicable to all GFE analysis.

5.2.6 Analyze the sample files.

5.3 Allelic Ladder Analysis Summary

This summary only appears if one or more of the allelic ladders have been identified as unacceptable by GMIDX.

Refer to the Interpretation Guidelines section for the review of allelic ladders.

GMIDX analysis requires the presence of at least one allelic ladder in each project. Allelic ladders with yellow and/or red quality indicators must be evaluated.

5.3.1 Select all ladder(s) that have either a yellow triangle or a red stop sign present in the Sizing Quality (SQ) column.

5.3.2 Select the “Size Match Editor” icon (icon with three red peaks) from the tool bar.

5.3.3 Review all of the ladder files verifying the GS600 peaks (See Figure 1 or 2 in Interpretation Guidelines).

5.3.4 Override the SQ of any acceptable ladder.

5.3.5 Verify all flagged allelic ladders have been labeled with the correct allele designations (See Figure 3 or 4 in Interpretation Guidelines). If a ladder requiring manual review is deemed acceptable, the composite genotype quality of the ladder must be overridden.

5.3.6 If an allelic ladder failed or is of poor quality (e.g., allelic ladder peaks are not labeled correctly or an extraneous peak is present within the allelic ladder), it may be removed from the project. Alternatively, the option to continue the analysis with at least one acceptable ladder may be selected.

5.4 Reviewing GeneMapper® ID-X Analysis Summary

Any sample or control listed as meeting all thresholds (designated as a green square) does not require a manual review.

Any control that is identified as not meeting one or more thresholds must be manually reviewed. Unacceptable controls must be left in the project for review during Primary Analysis.

5.5 Verifying the GS600 Internal Size Standard for Controls and FDDU Samples in GeneMapper® ID-X

Refer to the Interpretation Guidelines section for the review of GS600 size standards.

If any control contains a yellow triangle or a red stop sign in the *SQ* column, the size standard must be verified. If any sample contains a yellow triangle or a red stop sign in the *SQ* column, the size standard need only be verified if the flagged sample is being manually reviewed.

5.5.1 Select all control(s) that have either a yellow triangle or a red stop sign present in the *SQ* column.

5.5.2 If the flagged sample(s) are being manually reviewed, select all sample(s) that have either a yellow triangle or a red stop sign present in the *SQ* column.

5.5.3 Select the “Size Match Editor” icon (icon with three red peaks) from the tool bar.

5.5.4 Review all of the control/sample files verifying the GS600 peaks (See Figure 1 or 2 in Interpretation Guidelines).

5.5.5 Override the *SQ* of any acceptable control/sample.

5.6 Examining Data in GeneMapper® ID-X

5.6.1 Highlight the control(s)/sample(s) requiring manual review. Select the “Display Plots” icon (icon with multi-colored peaks) from the tool bar. This will open the *Samples Plot* window.

5.6.2 Peak labeling criteria are as follows:

- a. The 3130XL Analysis Method includes a global filter that removes labels from peaks that are less than 13% of the peak height of the largest allele present at each locus. The 3730 and 3500xL Analysis Methods include a global filter that removes labels from peaks that are less than 16% of the peak height of the largest allele present at each locus.
- b. Peaks that do not fall within an allelic category are labeled “OL” allele (i.e., off ladder allele).
- c. Peaks that are less than the Peak Amplitude Threshold (PAT) specified within the GMIDX Analysis Method Editor are not labeled.

5.6.3 Evaluate any control flagged by GMIDX. Refer to the Interpretation Guidelines section for Evaluation of Internal Size Standards, Allelic Ladders and Control Samples.

5.6.4 If a control requiring manual review is deemed acceptable, the composite genotype quality of the control must be overridden.

5.6.5 At the discretion of the Post PCR Reviewer, a general review may be performed by manually reviewing samples flagged by GMIDX to assess the overall quality of the data and whether the plate is acceptable and should be made available for Primary Analysis. If a general review is performed, proceed to step 5.6.11.

5.6.6 At the discretion of the Post PCR Reviewer, a detailed review may be performed in addition to the general review. The detailed review includes manually reviewing samples flagged by GMIDX to determine if individual samples require rework. Each locus will be designated with a colored heading (green, yellow, or red). Loci designated as either yellow or red must be manually reviewed. Loci designated as green do not need to be reviewed.

5.6.7 Unacceptable samples: Record the Plate Barcode, Sample ID, *Rework Reasons* and *Comments* (if applicable) for the sample(s) that are not acceptable for import into STACS and CODIS.

5.6.8 All unacceptable samples must either be designated for rework using the STACS automatic rework function or removed/deleted from the GMIDX Project.

- a. To delete a sample from the project, during the manual review, check the “Mark Sample for Deletion” box on the right side of the plot window. When the sample plot window is closed, GMIDX will ask for confirmation that the samples should be deleted.
- b. To designate a sample using the STACS automatic rework feature, the following information must be entered into the designated *User Defined* columns (e.g., UD1) of the *Samples* tab of the GMIDX Table: *Point/Action*, *Rework Reason*, *Comments* (optional) and *Next Run* (optional).

5.6.9 Select the “Genotypes” tab and verify that the following columns exist: *Sample Name*, *Marker*, *Allele 1*, *Allele 2*, *Allele 3*. If the STACS automatic rework functionality is being utilized, up to four *User Defined* columns (e.g., UD1) may also be included in the CODIS Table.

5.6.10 As applicable, select “Export Table” from the *File* menu in the GMIDX tool bar.

5.6.11 Save the GMIDX Project.

5.7 Exporting a GeneMapper® ID-X Project

5.7.1 In the “GeneMapper® ID-X Manager” select the project to be exported and click the “Export” button on the bottom of the window.

5.7.2 Browse to the location where the project will be exported to and click “Save”.

5.8 Completing Post PCR Review

5.8.1 If a general review was performed, a CODIS table is not generated. Indicate the result of the review in STACS as successful, failed or aborted. Comments must be entered for plates with process failed results.

5.8.2 If a detailed review was performed and a CODIS table was generated, import the file into STACS.

5.8.2.1 *Control Verification:* STACS compares the expected and current profiles for the Blood/Buccal Internal Standards (BIS), Positive, Negative, Combo, and AMPBLANK controls, (as applicable).

5.8.2.2 If an FDDU sample was deemed unacceptable and removed from the CODIS Table or designated for rework using the STACS automatic rework function, the *Sample ID* is displayed in the *Sample Rework* window.

5.8.2.3 As applicable, select a *Rework Point*, *Protocol*, *Rework Reason*, *Rework Comment*, and *Next Run* entry for all the samples in the *Sample Rework* window and complete the activity.

6 Primary Analysis (Review/Interpretation)

The genetic analysis data for all FDDU STR (Short Tandem Repeat) profiles must undergo primary analysis (interpretation) and secondary analysis (technical review) by a qualified FDDU Examiner.

Primary analysis may be performed by a FDDU Examiner at an offsite location that does not have access to the STACS software. In these instances, the FDDU Examiner must continue to follow all the steps in the procedures required for the review of FDDU data. However, the recording of the completion of primary analysis within STACS may be performed by the FDDU Examiner at a later date.

6.1 Within STACS, select the plate to be analyzed.

6.2 Analyzing a GeneMapper® ID-X Project

Refer to Section 5.2 for analyzing the sample files.

6.3 Allelic Ladder Analysis Summary

This summary only appears if one or more of the allelic ladders have been identified as unacceptable by GMIDX.

Refer to the Interpretation Guidelines sections for the review of allelic ladders.

GMIDX analysis requires the presence of at least one allelic ladder in each project. Allelic ladders with yellow and/or red quality indicators must be evaluated.

Refer to Section 5.3 for evaluating allelic ladders.

6.4 Reviewing GeneMapper® ID-X Analysis Summary

Any sample or control listed as meeting all thresholds (designated as a green square) does not require a manual review.

Any control that is identified as not meeting one or more thresholds must be manually reviewed. Unacceptable controls must be left in the project for review during Secondary Analysis.

6.5 Verifying the GS600 Internal Size Standard for Controls and FDDU Samples in GeneMapper® ID-X

Refer to the Interpretation Guidelines section for the review of GS600 size standards.

If any control contains a yellow triangle or a red stop sign in the *SQ* column, the size standard must be verified. If any sample contains a yellow triangle or a red stop sign in the *SQ* column, the size standard must only be verified if the flagged sample is being manually reviewed.

Refer to Section 5.5 for verifying the GS600 peaks for controls and/or samples.

6.6 Evaluation of Potential Crosstalk

Refer to the Interpretation Guidelines section for the review of potential crosstalk (CT).

6.6.1 Samples that appear to have CT peaks shall be reviewed to identify the source of the peak(s).

6.6.2 Select the “Samples” tab. For each sample and/or control identified, highlight the identified sample as well as the sample(s) in the adjacent capillaries based on capillary number.

6.6.3 Select the “Display Plots” icon (icon with multi-colored peaks) from the tool bar. Review the highlighted samples to ensure that crosstalk peaks have not been designated as true alleles.

6.7 Examining Data in GeneMapper® ID-X

6.7.1 Highlight the control(s)/sample(s) requiring manual review. Select the “Display Plots” icon (icon with multi-colored peaks) from the tool bar. This will open the *Samples Plot* window. .

6.7.2 Peak labeling criteria are as follows:

- a. The 3130XL Analysis Method includes a global filter that removes labels from

peaks that are less than 13% of the peak height of the largest allele present at each locus. The 3730 and 3500xL Analysis Methods include a global filter that removes labels from peaks that are less than 16% of the peak height of the largest allele present at each locus.

- b. Peaks that do not fall within an allelic category are labeled “OL” allele (i.e., off ladder allele).
- c. Peaks that are less than the Peak Amplitude Threshold (PAT) specified within the GMIDX Analysis Method Editor are not labeled.

6.7.3 Each locus will be designated with a colored heading (green, yellow, or red). Loci designated as either yellow or red must be manually reviewed. Loci designated as green do not need to be reviewed.

6.7.4 Evaluate any control flagged by GMIDX. Refer to the Interpretation Guidelines section for the Evaluation of Internal Size Standards, Allelic Ladders and Control Samples.

6.7.4.1 If a control requiring manual review is deemed acceptable, the composite genotype quality of the control must be overridden.

6.7.5 If necessary, evaluate the STR results for any FDDU sample(s) flagged by GMIDX. Refer to the Interpretation Guidelines sections for Evaluation of Peaks of Non-Genetic (Non-Allelic) Origin and Evaluation of FDDU DNA Samples.

6.7.5.1 At the discretion of a FDDU Examiner, samples flagged must either be manually reviewed (to determine if the samples are acceptable), designated for rework using the STACS automatic rework feature or removed/deleted from the GMIDX project.

6.7.5.2 The FDDU Examiner must review each flagged FDDU sample to examine the peak labels and edit the peak labels where/when necessary (e.g., labeling OL alleles, removing peak labels from a spike).

<u>Acceptable samples</u>	<p>Approved for import into STACS and CODIS. All labels must be removed (“Delete Alleles” option in GMIDX software) from peaks that are not attributable to DNA (e.g., spikes, bleed-through). Peak labels must also be removed from inconclusive loci. (i.e., one or more alleles < PIT (peak interpretation threshold)).</p> <p>NOTE: Hit confirmation samples and QA/QC samples with results \geq PAT (peak amplitude threshold) may be included in the CODIS table for import into STACS.</p>
<u>Off Ladder (OL) Alleles</u>	<p>If an off ladder allele is present in a sample, the peak label for the allele must be manually edited in GMIDX to include the letters “OL” before the number designation for the allele (e.g., OL>10).</p>

	NOTE: Hit confirmation samples and QA/QC samples with OL alleles do not need to be labelled with a “OL”.
<u>Peak Height Ratio (PHR) and Allele Imbalance (AI)</u>	<p>If the peak height ratio for alleles at a heterozygote loci is <60% for Identifiler or <50% for Identifiler Direct and Globalfiler Express, the peak label for the allele with lower RFU (relative fluorescence unit) value may be manually edited in GMIDX to include the letters “PH” (peak height) or “AI” (allele imbalance) before the number designation for the allele (e.g., AI23).</p> <p>NOTE: Hit confirmation samples and QA/QC samples with PHR or AI do not need to be labelled with a “PH” or “AI”.</p>
<u>Tri-allelic (TRI) Patterns</u>	If a sample has a tri-allelic pattern at a locus, the allele values that meet the peak interpretation threshold should be included in the CODIS Table and imported into STACS.
<u>Unacceptable samples</u>	Record the Plate Barcode, Sample ID, <i>Rework Reasons</i> , and <i>Comments</i> (if applicable) for the sample(s) that are not acceptable for import into STACS and CODIS. For samples flagged by GMIDX, but not manually reviewed, it is not necessary to make note of each Sample ID and a general <i>Rework Reason</i> (e.g., GMIDX Rework) may be recorded for all the samples.

6.7.5.3 If a sample requiring manual review is deemed acceptable, the composite genotype quality of the sample must be overridden.

6.7.5.4 All unacceptable samples must either be designated for rework using the STACS automatic rework function or removed/deleted from the GMIDX Project. Samples flagged by GMIDX, but not manually reviewed, are unacceptable and must be removed/deleted from the GMIDX Project or designated for rework.

- a. To delete a sample from the project, during the manual review, check the “Mark Sample for Deletion” box on the right side of the plot window. When the sample plot window is closed, GMIDX will ask for confirmation that the samples should be deleted.
- b. To designate a sample using the STACS automatic rework feature, the following information must be entered into the designated user defined columns (e.g., UD1) of the *Samples* tab of the GMIDX table: *Point/Action*, *Rework Reason*, *Comments* (optional) and *Next Run* (optional).

6.7.6 At the discretion of the FDDU Examiner, a CODIS Table may be generated at the completion of Primary Analysis for import into STACS resulting in sample(s) being sent back for rework. If a CODIS Table is not generated, proceed to step 6.7.9.

6.7.7 Select the “Genotypes” tab and verify that the following columns exist: *Sample Name*, *Marker*, *Allele 1*, *Allele 2*, *Allele 3*. If the STACS automatic rework function is being utilized, up

to four *User Defined* columns (e.g., UD1) may also be included in the CODIS Table.

6.7.8 As applicable, select “Export Table” from the *File* menu in the GMIDX tool bar.

6.7.9 Save the GMIDX Project.

6.8 Exporting a GeneMapper® ID-X Project

6.8.1 In the “GeneMapper® ID-X Manager” select the project to be exported and click the “Export” button on the bottom of the window.

6.8.2 Browse to the location where the project will be exported to and click “Save”.

6.9 Completing Primary Analysis

6.9.1 If a CODIS Table was not generated, indicate the result of the Primary Analysis in STACS as successful, failed or aborted. Comments must be entered for plates with processed failed results.

6.9.2 If a CODIS Table was generated, import the file into STACS.

6.9.2.1 *Control Verification:* STACS compares the expected and current profiles for the Blood/Buccal Internal Standards (BIS), Positive, Negative, Combo, and AMPBLANK controls, (as applicable).

6.9.2.2 If an FDDU sample was deemed unacceptable and removed from the CODIS Table or designated for rework using the STACS automatic rework function, the *Sample ID* is displayed in the *Sample Rework* window.

6.9.2.3 As applicable, select a *Rework Point*, *Protocol*, *Rework Reason*, *Rework Comment*, and *Next Run* entry for all the samples in the *Sample Rework* window and complete the activity.

7 Secondary Analysis (Technical Review)

The FDDU Examiner that performs the secondary analysis (technical review) must be different from the FDDU Examiner that performed the primary analysis (interpretation).

Secondary analysis may be performed by a FDDU Examiner at an offsite location that does not have access to the STACS software. In these instances, the FDDU Examiner must continue to follow all the steps in the procedures required for the review of FDDU data. However, the recording of the completion of secondary analysis within STACS and the import of the resulting CODIS Table may be performed by the FDDU Examiner at a later date.

7.1 Within STACS, select the plate to be analyzed.

7.2 Importing and Reviewing a GeneMapper® ID-X Project

7.2.1 Open the GMIDX software, if not already open and select “GeneMapper® ID-X Manager” from the *Tools* menu on the tool bar.

7.2.2 From the *Projects* tab, click the “Import” button on the bottom of the window.

7.2.3 Browse to the project that is to be imported. Highlight the project and select “Import”. Select “GeneMapper® ID-X Security Group” and select “OK”.

NOTE: If the project already exists in the GMIDX database, it does not need to be reimported.

7.3 Click on the “Analysis Summary” tab and verify that all ladders, controls and/or samples flagged as “One or more thresholds not met” (designated by a red stop sign CGQ [Composite Genotype Quality]) have either been:

- (a) Reviewed and accepted following an edit and CGQ override in GMIDX (designated by a green stop sign CGQ); or
- (b) Designated for rework using the STACS automatic rework function; or
- (c) Deleted from the GMIDX project.

NOTE: Unacceptable controls (designated by a red stop sign) should have been left in the project during Primary Analysis so they are available for review by the FDDU Examiner performing Secondary Analysis.

7.4 Click on the “Samples” tab, then “Project” in the navigation panel on the left side of the screen. This will bring up an unfiltered list of all samples in the project.

7.5 Evaluate the GS600 Internal Size Standards for any ladder, control, and/or sample where the sizing quality was overridden during post PCR review or primary analysis as designated by a green check in the *SQO* (Sizing Quality Overridden) column. Refer to the Interpretation Guidelines section for the review of GS600 size standards.

7.6 Evaluate the electronic data in GMIDX using the *Raw Data* tab for each Negative or Combo control and Amplification Blank, if applicable, to verify the presence of a primer peak in the raw data.

7.7 Select the table setting “View CGQ Overrides”. This will display a filtered list of all ladders/controls/samples where the CGQ was overridden. This view only displays samples where the CGQ was overridden, (e.g., a sample containing a tri-allele at a locus was deemed acceptable and the marker overridden during Primary Analysis).

7.7.1 Evaluate the STR results for all ladders/controls/samples that are listed. Refer to the Interpretation Guidelines sections for Evaluation of Peaks of Non-Genetic (Non-Allelic) Origin and Evaluation of FDDU DNA Samples.

7.7.2 The FDDU Examiner must review each FDDU sample and/or control listed in the table “View CGQ Overrides” to examine the peak labels and review the previously recorded results to

ensure that he/she is in agreement with the interpretations made during Primary Analysis.

7.8 Select the table setting “View Edited Samples”. This will display a filtered list of all ladders/controls/samples where an edit was made to a specific allele call, (i.e., removal of a pull-up peak or renaming of an off-ladder allele during Primary Analysis).

7.8.1 Evaluate the STR results for all ladders/controls/samples that are listed. Refer to the Interpretation Guidelines sections of Evaluation of Peaks of Non-Genetic (Non-Allelic) Origin and Evaluation of FDDU DNA Samples.

7.8.2 The FDDU Examiner must review each FDDU ladder/control/sample listed in the table “View Edited Samples” to examine the peak labels and review the previously recorded results to ensure that he/she is in agreement with the interpretations made during Primary Analysis.

7.9 Evaluate any buccal samples and/or controls that were previously recorded during Primary Analysis as having crosstalk peaks present. Refer to the Interpretation Guidelines section for the review of potential crosstalk.

7.10 **Unacceptable Samples:** Verify that the previously recorded Post PCR Review or Primary Analysis results include a list of each Sample ID removed from the GMIDX Project and a corresponding *Rework Reason* and *Comment* (if applicable). For samples flagged by GMIDX, but not manually reviewed, it is not necessary to record each Sample ID and a general *Rework Reason* (e.g., GMIDX Rework) may be recorded for all the samples.

7.11 At the discretion of the FDDU Examiner performing Secondary Analysis, additional FDDU samples deemed unacceptable may be designated for rework using the STACS automatic rework feature or removed/deleted from the project. Samples flagged by GMIDX, but not manually reviewed, are unacceptable and must be removed/deleted from the GMIDX Project.

7.12 If there are interpretation discrepancies between the primary and secondary analysis, they must be resolved before the *Secondary Analysis* can be completed and the FDDU STR profiles are approved for import into STACS and CODIS.

7.13 Completing Secondary Analysis

7.13.1 Following completion of the secondary analysis (technical review), the FDDU Examiner must generate a CODIS table.

7.13.1.1 Select the “Genotypes” tab and verify that the following columns exist: *Sample Name*, *Marker*, *Allele 1*, *Allele 2*, and *Allele 3*. If the STACS automatic rework function is being utilized, up to four *User Defined* columns (e.g., UD1) may also be included in the CODIS table.

7.13.1.2 Select “Export Table” from the *File* menu in the GMIDX tool bar.

7.13.1.3 If any modifications were made to the GMIDX Project, save the project and use the *GeneMapper® ID-X Manager* Export option to export the project into the existing local project

folder that was copied from the STACS network at the beginning of Secondary Analysis.

7.13.2 Import the CODIS Table into STACS.

7.13.2.1 Control Verification: STACS compares the expected and current profiles for the Blood/Buccal Internal Standards (BIS), Positive, Negative, Combo, and AMPBLANK controls (as applicable). The results are displayed in the *Control Verifications Results* window. Control profiles that fail to exhibit the expected DNA typing results appear in red; failures must be recorded.

7.13.2.2 If an FDDU sample was deemed unacceptable and removed from the CODIS Table or designated for rework using the STACS automatic rework function, the *Sample ID* is displayed in the *Missing Rework* list.

7.13.3 As applicable, select a rework *Action*, *Protocol*, *Reason*, *Comment*, and *Next Run* for all the samples in the *Missing Rework* window.

7.13.4 As applicable, review the results displayed on each STACS form displayed (e.g., Validation Rework, Invalid Allele, Hit Confirmation, Mismatch) and take the appropriate action to complete the activity.

8 Interpretation Guidelines

8.1 Evaluation of Internal Size Standards, Allelic Ladders and Control Samples

8.1.1 Internal Size Standard: GS600 [LIZ]

Verify the correct fragments are captured and have been assigned the correct size values for all FDDU samples, controls, and allelic ladders. See Figures 1 and 2.

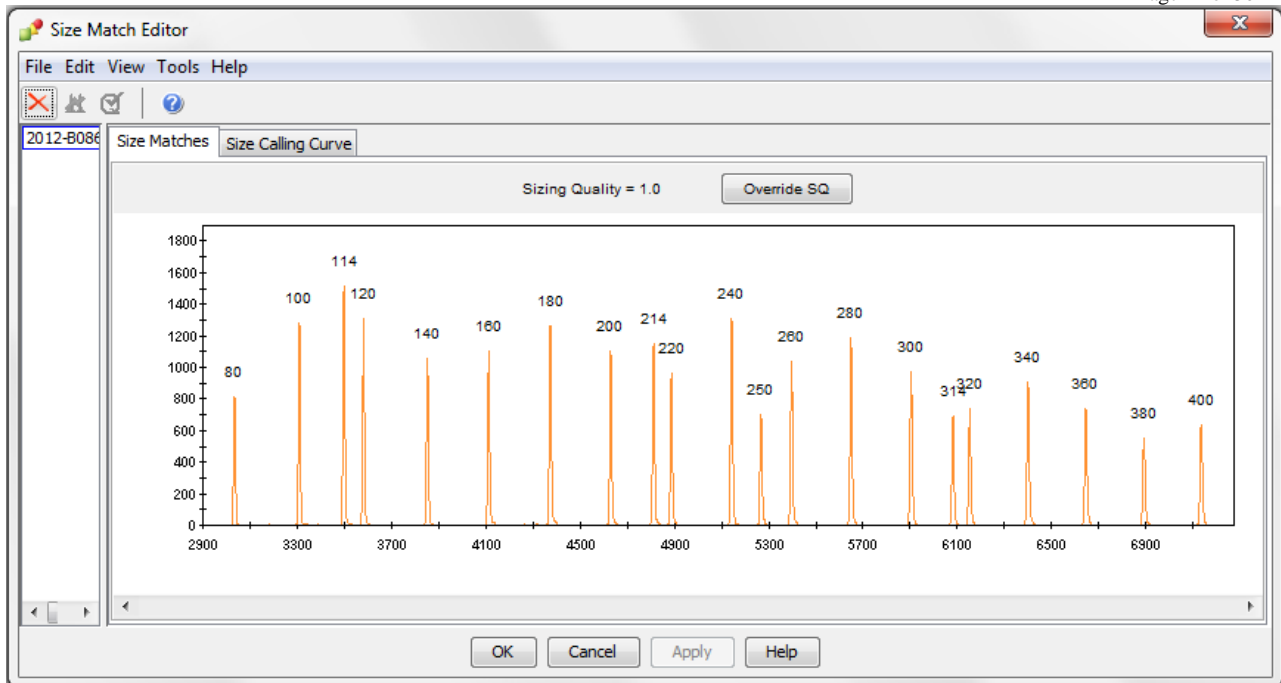


Figure 1 – GS600 Size Standard (ID and IDD)

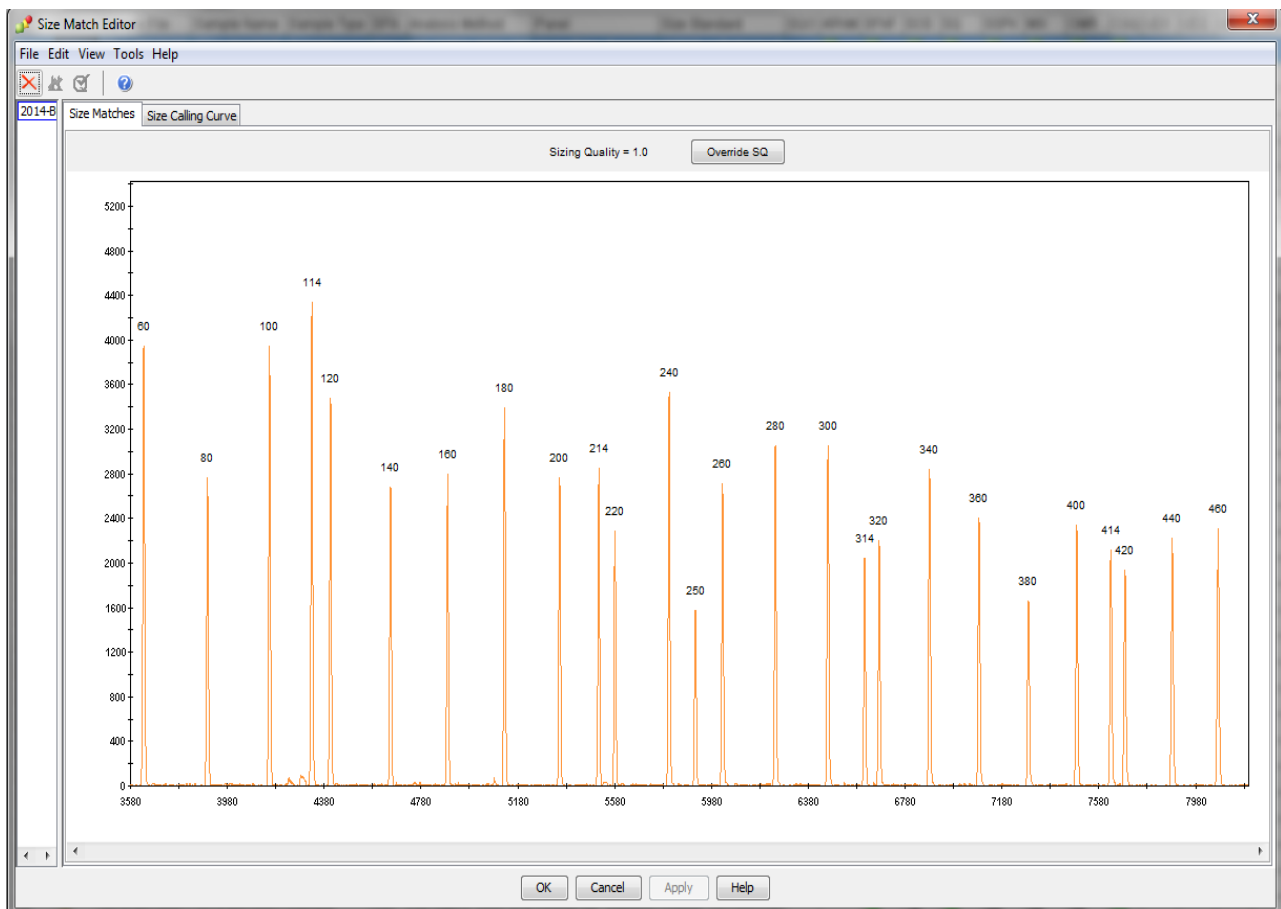


Figure 2 – GS600 v2.0 Size Standard (GFE)

8.1.2 Allelic Ladders

The Identifiler, Identifiler Direct and/or Globalfiler Express Allelic Ladder, which is run separately from any sample(s) or control(s), is used by the GMIDX software as a reference for designating alleles. Each allelic ladder is a kit reagent that consists of amplified allelic fragments of known size and repeat content. These ladders do not contain all possible alleles that could be detected at an individual STR locus (i.e., off-ladder alleles). Any allelic ladder(s) used for comparison and assignment of allele designations must exhibit the correct allele designations in GMIDX. See Figure 3 and 4.

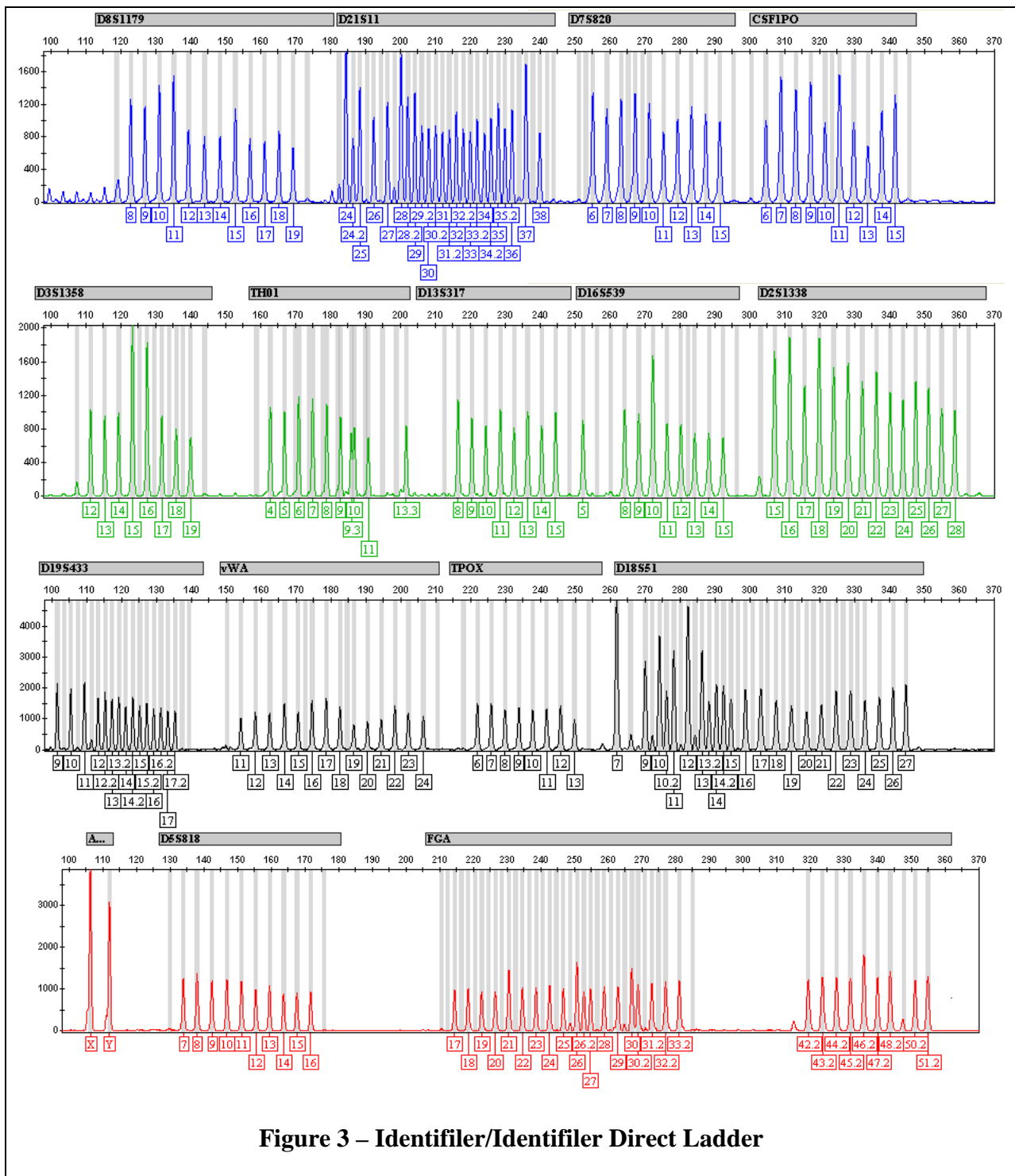


Figure 3 – Identifiler/Identifiler Direct Ladder

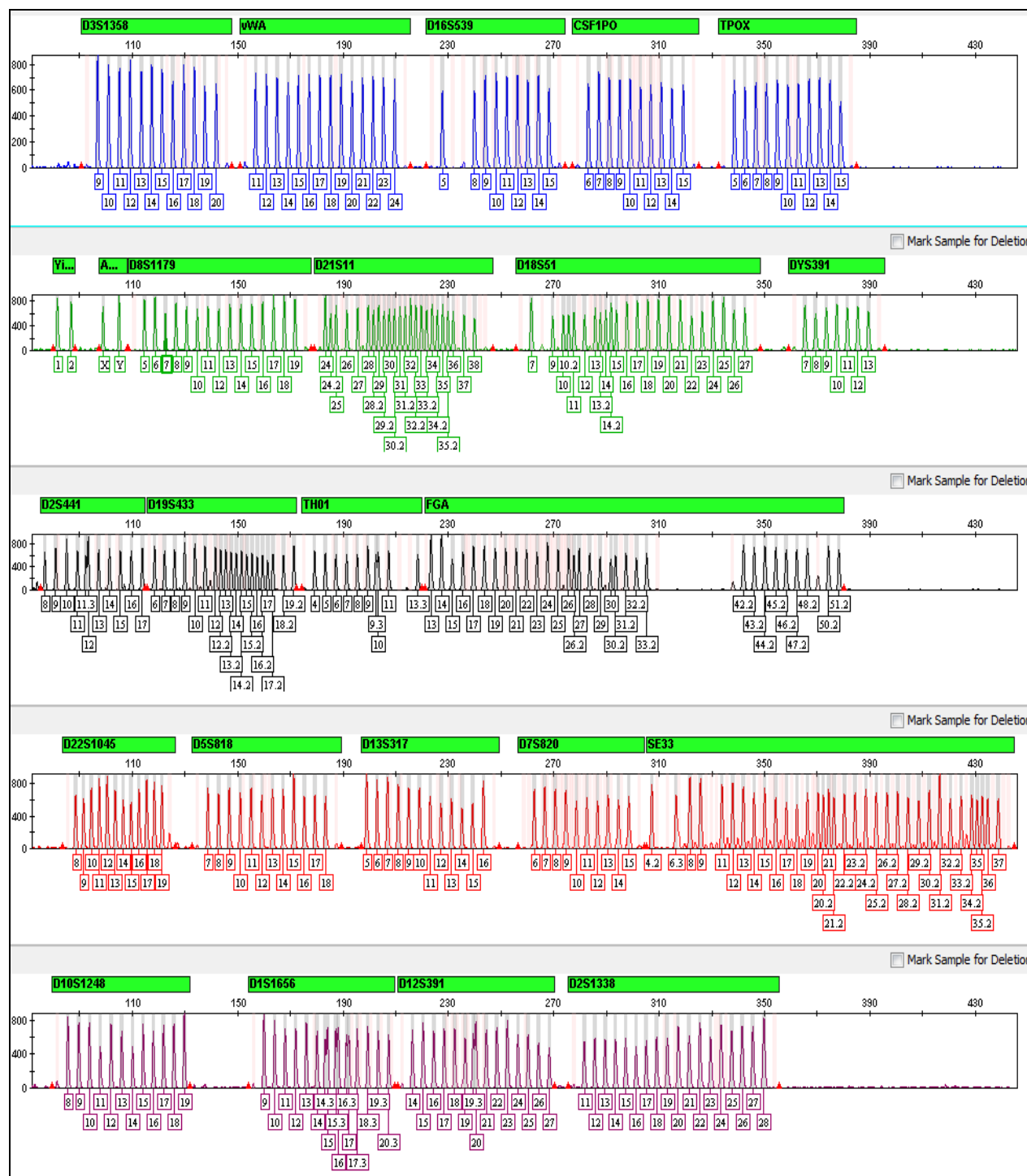


Figure 4 – GlobalFiler Express Ladder

8.1.3 Blood/Buccal Internal Standard (BIS)

A Blood/Buccal Internal Standard is a positive control for both the STR loci and amelogenin sex typing locus. Two (2) BIS controls must be included with each 96 Well plate of FDDU samples punched. The BIS control is processed through the same extraction and/or amplification and electrophoretic typing procedure as the FDDU samples and is used to evaluate the performance of the entire FDDU DNA analysis procedure. Any combination of BIS controls from approved donors may be used. The DNA typing results for the BIS controls are verified by STACS during Secondary Analysis.

8.1.3.1 At minimum, one BIS control included on each plate must yield acceptable DNA typing results that exhibit all the expected allelic peaks (\geq PAT) and must not exhibit any extraneous allelic peaks.

8.1.3.2 If one BIS control is acceptable and exhibits the expected DNA typing results, but the second BIS control yields unacceptable results (e.g., injection failure, $<$ PAT, loss of resolution, off scale), no action is required. However, it should be verified that all loci with designated allele values are consistent with the expected STR typing results for the BIS control by leaving the BIS control in the GMIDX project and allowing the type to be verified by STACS during Secondary Analysis.

8.1.3.3 If both BIS controls yield unacceptable DNA typing results, the following steps must be taken (as applicable):

- a. If there appears to be an injection or electrophoretic problem (e.g., injection failure, loss of resolution, off scale data, excessive bleed-through), re-inject the BIS control(s), or, if necessary, re-inject the entire plate.¹ If re-injection of the BIS control(s) does not resolve the problem, all FDDU samples associated with the BIS control(s) must be re-worked.
- b. If there does not appear to be an injection or electrophoretic problem, all FDDU samples associated with the BIS control(s) must be re-worked.²

8.1.3.4 If one or both BIS controls are acceptable, but fail to exhibit the expected DNA typing result, the following steps must be taken (as applicable):

- a. If there appears to be an injection or electrophoretic problem (e.g., mobility shift, loss of resolution), re-inject the BIS control(s), or, if necessary, re-inject the entire plate. If re-injection of the BIS control(s) does not resolve the problem, all FDDU

¹ If the BIS control(s) exhibited off scale data, they may be re-injected with less DNA (i.e., by reducing the injection time).

²As used in this procedure, “re-worked” refers to sending the sample(s) back to the STaCS Plate Creation module so they can be assigned to a new plate and re-processed through the entire FDDU DNA Analysis procedure (e.g., FTA Extraction, STR amplification, Capillary Electrophoresis).

samples associated with the BIS control(s) must be re-worked.

- b. If there does not appear to be an injection or electrophoretic problem, all FDDU samples associated with the BIS control(s) must be re-worked.

It should be verified that all loci with designated allele values are consistent with the expected STR typing results for the BIS control by leaving the BIS control in the GMIDX project and allowing the type to be verified by STACS during Secondary Analysis.

8.1.4 Negative Control (NEGATIVE)

Two (2) Negative controls must be included with each 96-Well plate amplified with Identifiler. The Negative control contains all of the reagents used in the pre-amplification process, minus a sample punch, and is processed through the same plate preparation, extraction and/or amplification and electrophoretic typing procedures as the FDDU samples. Because the Negative control contains all of the pre-amplification reagents, as well as the amplification reagents, it functions as a reagent blank control for the detection of potential DNA contaminant(s) that may have been introduced in the pre-amplification and/or amplification reagents. The expected outcome is that no DNA typing results are obtained for the Negative control(s). The detection of allelic peak(s) in the Negative control(s) is indicative of the potential presence of adventitious DNA in the reagents and/or supplies used during pre-amplification and/or DNA amplification. The source of the adventitious DNA can be non-amplified DNA or amplified PCR product.

8.1.4.1 If one Negative control is acceptable and the second Negative control exhibits unacceptable results indicative of an injection or electrophoretic problem (e.g., injection failure, loss of resolution) no action is required.

8.1.4.2 If a Negative control exhibits a peak(s) \geq PAT within the expected size range of the AmpF/STR loci that are not attributable to a spike or other artifact (e.g., raised baseline, dissociated primer dye, crosstalk), all FDDU samples extracted and/or amplified with the reagents contained in the Negative control must be re-worked.

8.1.4.3 At the discretion of a FDDU Examiner, Negative controls containing spikes or other artifacts (e.g., raised baseline, dissociated primer dye) within the expected size range of the AmpF/STR loci may be re-injected. Spikes or other artifacts that fall outside the expected size range are not significant.

- a. Subsequent injections of the Negative control that also exhibit spikes need not be re-injected if the original and subsequent spikes have different base pair sizes.
- b. If there is more than one Negative control included with the FDDU samples and: 1) Both controls have spikes, but they have different base pair sizes -OR- 2) One control has a spike that is not present in the other Negative control(s); the samples need not be re-injected.

8.1.4.4 For each electrophoretic run of a Negative control, the electronic data must be reviewed by a FDDU Examiner in GMIDX to verify the presence of a primer peak in the raw data.

8.1.5 Combo Control (NEGATIVE)

Two (2) Combo controls, designated as a NEGATIVE in STACS, must be included with each 96-Well plate amplified with Identifiler Direct or Globalfiler Express. The Combo control is a combination of the Negative and Amplification Blank controls for the amplification process. This control contains all the reagents used in the pre-amplification and amplification process minus a sample punch and is processed through the same plate preparation, amplification and electrophoretic typing procedures as the FDDU samples. The Combo control functions as a reagent blank control for detection of potential DNA contamination(s) that may have been introduced in the pre-amplification or amplification reagents. The expected outcome is that no DNA typing results are obtained for the Combo control(s). The detection of allelic peak(s) in the Combo control(s) is indicative of the potential presence of adventitious DNA in the reagents and/or supplies used during pre-amplification and/or DNA amplification. The source of the adventitious DNA can be non-amplified DNA or amplified PCR product.

8.1.5.1 If one Combo control is acceptable and the second Combo control exhibits unacceptable results indicative of an injection or electrophoretic problem (e.g., injection failure, loss of resolution) no action is required.

8.1.5.2 If a Combo control exhibits a peak(s) \geq PAT within the expected size range of the AmpF/STR loci that are not attributable to a spike or other artifact (e.g., raised baseline, dissociated primer dye, crosstalk), all FDDU samples extracted and/or amplified with the reagents contained in the Combo control must be re-worked.

8.1.5.3 At the discretion of a FDDU Examiner, Combo controls containing spikes or other artifacts (e.g., raised baseline, dissociated primer dye) within the expected size range of the AmpF/STR loci may be re-injected. Spikes or other artifacts that fall outside the expected size range are not significant.

- a. Subsequent injections of the Combo control that also exhibit spikes need not be re-injected if the original and subsequent spikes have different base pair sizes.
- b. If there is more than one Combo control included with the FDDU samples and: 1) Both controls have spikes, but they have different base pair sizes -OR- 2) One control has a spike that is not present in the other Combo control(s); the samples need not be re-injected.

8.1.5.4 For each electrophoretic run of a Combo control, the electronic data must be reviewed by a FDDU Examiner in GMIDX to verify the presence of a primer peak in the raw data.

8.1.6 Amplification Blank (AMPBLANK)

Two (2) Amplification Blanks must be included with each 96-Well plate amplified with Identifiler.

The Amplification Blank contains all of the chemical components required for the amplification of DNA, minus an FTA punch, and is processed through the same amplification and electrophoretic typing procedures as the FDDU samples. The Amplification Blank functions as a reagent blank control for the detection of potential DNA contaminant(s) that may have been introduced in the amplification reagents. The expected outcome is that no DNA typing results are obtained for the Amplification Blank(s). The detection of allelic peak(s) in the Amplification Blank(s) is indicative of the potential presence of adventitious DNA in the reagents and/or supplies used during DNA amplification. The source of the adventitious DNA can be non-amplified DNA or amplified PCR product.

8.1.6.1 If one Amplification Blank is acceptable and the second Amplification Blank exhibits unacceptable results indicative of an injection or electrophoretic problem (e.g., injection failure, loss of resolution) no action is required.

8.1.6.2 If an Amplification Blank exhibits a peak(s) \geq PAT within the expected size range of the AmpF/STR[®] loci that are not attributable to a spike or other artifact (e.g., raised baseline, dissociated primer dye, crosstalk), all FDDU samples extracted and amplified with the reagents contained in the Amplification Blank must be re-worked.

8.1.6.3 At the discretion of a FDDU Examiner, Amplification Blanks containing spikes or other artifacts (e.g., raised baseline, dissociated primer dye) within the expected size range of the AmpF/STR[®] loci may be re-injected. Spikes or other artifacts that fall outside the expected size range are not significant.

- a. Subsequent injections of the Amplification Blank that also exhibit spikes need not be re-injected if the original and subsequent spikes have different base pair sizes.
- b. If there is more than one Amplification Blank included with the FDDU samples and:
1) Both controls have spikes, but they have different base pair sizes -OR- 2) One control has a spike that is not present in the other Amplification Blank(s); the samples need not be re-injected.

8.1.6.4 For each electrophoretic run of an Amplification Blank, the electronic data must be reviewed by a FDDU Examiner in GMIDX to verify the presence of a primer peak in the raw data.

8.1.7 Positive Control (If applicable)

Control DNA 9947A (Promega or Applied Biosystems) is a positive amplification control for both the short tandem repeat (STR) loci and amelogenin sex typing locus. Two (2) Positive controls must be included with each 96-Well plate amplified with Identifiler. The Positive control is processed through the same amplification and electrophoretic typing procedures as the FDDU samples and is used to evaluate the performance of these procedures.

8.1.7.1 At minimum, one 9947A control included on each plate must yield acceptable DNA typing results that exhibit all the expected allelic peaks ($>$ PAT) and must not exhibit any extraneous allelic peaks.

8.1.7.2 If one 9947A control is acceptable and exhibits the expected DNA typing results, but the second 9947A control yields unacceptable results (e.g., injection failure, < PAT, loss of resolution, off scale, etc.), no action is required. However, it should be verified that all loci with designated allele values are consistent with the expected STR typing results for the 9947A control by leaving the positive control in the GMIDX project and allowing the type to be verified by STACS during Secondary Analysis.

8.1.7.3 If both 9947A controls yield unacceptable DNA typing results, the following steps must be taken (as applicable):

- a. If there appears to be an injection or electrophoretic problem (e.g., injection failure, loss of resolution, off scale data, excessive bleed-through), re-inject the 9947A control(s), or, if necessary, re-inject the entire plate. If re-injection of the 9947A control(s) does not resolve the problem, all FDDU samples associated with the 9947A control(s) must be re-worked.
- b. If there does not appear to be an injection or electrophoretic problem, all FDDU samples associated with the 9947A control(s) must be re-worked.

8.1.7.4 If one or both 9947A controls are acceptable, but fail to exhibit the expected DNA typing result, the following steps must be taken (as applicable):

- a. If there appears to be an injection or electrophoretic problem (e.g., mobility shift, loss of resolution), re-inject the 9947A control(s), or, if necessary, re-inject the entire plate. If re-injection of the 9947A control(s) does not resolve the problem, all FDDU samples associated with the 9947A control(s) must be re-worked.
- b. If there does not appear to be an injection or electrophoretic problem, all FDDU samples associated with the 9947A control(s) must be re-worked.

8.2 Evaluation of Peaks of Non-Genetic (Non-Allelic)³ Origin

Non-genetic data may be undesired PCR products (e.g., stutter and non-template dependent nucleotide addition), analytical artifacts (e.g., spikes, raised baseline), instrumental limitations (e.g., pull-up, bleed-through, and crosstalk), or be introduced into the process (e.g., disassociated primer dye). The various types, and evaluation, of these non-genetic peaks are described below.

8.2.1 Stutter

Stutter is a minor peak product that is inherent to the PCR amplification of repetitive DNA sequences (e.g., STR loci). Stutter peaks originate as the template DNA is being copied during the

³Non-genetic or Non-specific peaks are those that do not represent (i.e., do not have their origin in) allelic STR information.

PCR reaction and do not constitute an individual's genotype. Stutter is most often observed as a minor PCR product one repeat unit smaller (e.g., -4 bp in a tetranucleotide STR locus) than the source allelic peak. Stutter is expressed as a percentage relative to the source allelic peak (i.e., % Stutter). The GMIDX analysis methods have a 20% filter that removes any stutter peaks less than 20% of the corresponding allele. At the discretion of a FDDU Examiner, FDDU samples exhibiting stutter >20% at a locus may be re-worked.

8.2.2 Non-Template Dependent Nucleotide Addition

Taq DNA Polymerase is known to add an additional nucleotide (i.e., typically "A") to the 3' ends of double-stranded PCR products in a non-template dependent manner. This phenomenon is called "adenylation" and results in the generation of the "plus-A" fragment. This plus-A fragment is the allele (N) produced by the PCR amplification. A minority of amplified fragments do not have this additional nucleotide added, these "minus-A" fragments are one bp shorter (N-1) than the allelic fragment. The generation of minus-A fragments is sequence dependent and thus kit and locus specific. Minus-A is expressed as a percentage relative to the allelic peak (i.e., % minus-A). At the discretion of a FDDU Examiner, FDDU samples exhibiting minus-A >13% on the 3130xl or >16% on the 3730 or 3500xL at a locus may be re-worked.

8.2.3 Spikes

8.2.3.1 Spikes are non-specific, non-reproducible peaks that may result during capillary electrophoresis. Spikes may be caused by electrical fluctuations in the power source, urea crystals, or bubbles in the polymer. Spikes are generally detected in two or more colors and size within ± 0.13 bp (\pm approximately one scan unit) of each other; however, spikes that occur in only one color have been observed.

8.2.3.2 At the discretion of a FDDU Examiner, FDDU samples exhibiting spikes within the expected size range of the AmpF/STR[®] loci may be re-injected or re-worked. However, if a spike occurs at or sufficiently close to an internal size standard peak or an allelic peak such that sizing and/or allelic designation is affected, the FDDU sample(s) must be re-injected or re-worked. Subsequent runs of the sample that also exhibit spikes need not be re-injected or re-worked if the original and subsequent spikes have different base pair sizes.

8.2.4 Raised Baseline

Raised baseline appears in an electropherogram as a non-specific elevation of the baseline (horizontal axis) between one or more peaks. This elevation may result from excessive template DNA or be instrument related. At the discretion of a FDDU Examiner, samples exhibiting a raised baseline may be re-injected or re-worked.

8.2.5 Pull-Up

The excessive fluorescence intensity associated with an off scale peak may result in the appearance of a lower intensity peak that is generally observed in the color channels spectrally adjacent to that of the off scale peak. The resulting artifact is called a pull-up peak and typically

sizes within ± 0.25 bp (\pm approximately two scan units) of its source peak. At the discretion of a FDDU Examiner, samples exhibiting pull-up may be re-injected with less DNA (i.e., by reducing the injection time) or re-worked.

8.2.6 Bleed-Through

A bleed-through peak is similar to a pull-up peak (i.e., it appears in a spectrally adjacent color and sizes within ± 0.25 bp of its source peak). However, the source of the bleed-through peak is not off scale data. At the discretion of a FDDU Examiner, samples exhibiting bleed-through may be re-injected or re-worked.

8.2.7 Dissociated Primer Dye

Dye peaks may result from dye that has become dissociated from the primers present in some lots of the AmpF/STR[®] kits and migrate independently during capillary electrophoresis. Since the dissociated dye originates from a kit reagent, dye peaks that exceed the GMIDX PAT may be detected in any sample or control. Dye peaks possess the spectrum of one of the dyes in the AmpF/STR[®] kit(s) and a broader morphology than that of allelic peaks. At the discretion of a FDDU Examiner, samples exhibiting dye peaks may be re-injected or re-worked.

8.2.8 Crosstalk

Crosstalk between adjacent capillaries can be observed when one capillary contains a low quantity of DNA and an adjacent capillary contains a high quantity of DNA that results in a large amount of fluorescence being captured by the CCD (Charge Coupled Device) camera. The high level of fluorescence and the proximity of the capillaries can cause a low level of fluorescence to appear in an adjacent capillary. The level of CT can be higher for buccal samples than for blood samples. At the discretion of an FDDU Examiner, samples exhibiting crosstalk may be re-injected or re-worked.

8.3 Evaluation of FDDU DNA Samples

8.3.1 Peak Interpretation Threshold (PIT) and Peak Amplitude Threshold (PAT)

8.3.1.1 The PIT for FDDU samples is ≥ 150 RFU for Identifiler and Identifiler Direct or ≥ 175 RFU for Globalfiler Express.⁴ STR loci with peaks $<$ PIT are considered inconclusive and ineligible for entry into STACS and CODIS.

8.3.1.2 The PAT for FDDU samples is ≥ 50 RFU for Identifiler and Identifiler Direct or ≥ 75 RFU for Globalfiler Express. Peaks $<$ PAT are not labeled during GMIDX analysis.

⁴The 3130XL Analysis Method includes a global filter that removes labels from peaks that are less than 13% of the peak height of the largest allele present at each locus. The 3730 and 3500xL Analysis Methods include a global filter that removes labels from peaks that are less than 16% of the peak height of the largest allele present at each locus.

8.3.1.3 If labeled peaks \geq PAT indicate the potential presence of a mixture or contamination (adventitious DNA), the affected sample(s) and/or control(s) must be re-worked.

8.3.2 Excessive DNA Template and Off Scale Samples

8.3.2.1 An excessive amount of template DNA may result in the appearance of off scale peaks. These samples may also exhibit raised baseline, pull-up peaks, elevated stutter and/or incomplete non-template-dependent nucleotide addition (-A) peaks, and/or non-specific peaks in one or more colors. Samples with an excessive amount of DNA template may also exhibit the similar characteristics, but in the absence of off scale peaks.

8.3.2.2 At the discretion of a FDDU Examiner, samples that exhibit the characteristics described above (both in the presence and absence of off scale peaks), may be re-injected with less DNA (i.e., by reducing the injection time) or re-worked.

8.3.3 Interpretation of Single Source FDDU DNA Samples

8.3.3.1 A sample is considered to have originated from a single individual if only one or two alleles are present at all loci for which typing results were obtained,⁵ and the peak height ratios for all heterozygous loci are within the expected value⁶ (see *Peak Height Ratios and Allele Imbalance* section).

8.3.3.2 If a sample exhibits more than two alleles at two or more loci and/or the peak height ratios between the allelic peaks for more than one locus are below the expected value, the sample shall be handled as a possible mixture and re-worked.

8.3.4 Peak Height Ratios and Allele Imbalance

For alleles at a heterozygote locus, Peak Height Ratios (PHR) are determined by dividing the peak height of the allele with the lower RFU value by the peak height of the allele with the higher RFU value, and then multiplying this value by 100 to express the PHR as a percentage. The expected PHR for a heterozygote locus is $\geq 60\%$ for Identifiler or $\geq 50\%$ for Identifiler Direct and GlobalFiler Express.

8.3.4.1 Allele (peak height) imbalance may be seen in the typing results from a single individual due to elevated stutter, primer binding site variants that result in attenuated amplification of one allele of a heterozygous pair, tri-allelic patterns, etc. Samples exhibiting a PHR $< 60\%$ for Identifiler or $< 50\%$ for Identifiler Direct and Globalfiler Express at more than one of the CODIS Core Loci must be re-worked for confirmation. At the discretion of a FDDU

⁵Individuals can display three allelic peaks at a locus (see *Tri-allelic Patterns* section). Samples in which three allelic peaks at a locus have been observed and confirmed may be concluded to be single-source if no other typing results indicate the presence of a mixture.

⁶Allele (peak height) imbalances may be seen in the typing results from a single individual (see *Peak Height Ratios and Allele Imbalance* section). Samples in which allele imbalance at a locus has been observed and confirmed may be concluded to be single-source if no other typing results indicate the presence of a mixture.

Examiner, samples exhibiting a PHR <60% for Identifiler or <50% for Identifiler Direct and Globalfiler Express at one of the CODIS Core Loci may be re-worked for confirmation. At the discretion of a FDDU Examiner, a non-CODIS Core locus exhibiting a PHR <60% or <50% (as applicable) may be considered inconclusive and ineligible for entry into STACS and CODIS.

8.3.5 Off Ladder (OL) Alleles

8.3.5.1 Using alleles that are present in the allelic ladder, GMIDX creates allele size categories that are centered on the size of the allelic ladder peaks. Additionally, GMIDX creates categories for “virtual” alleles (ones not physically present in the allelic ladder and thus have no corresponding peaks in the electropherogram of the allelic ladder) at some loci. Off-ladder (OL) alleles are those DNA fragments that fail to size within an allele category.

8.3.5.2 OL alleles that fall between alleles physically present within the allelic ladder will be designated in accordance with guidelines of the International Society for Forensic Haemogenetics. To determine the allelic designation, OL peaks are converted to size in base pairs (bp) and compared to the size of the flanking ladder alleles. If the OL allele is not an integer tetranucleotide repeat (e.g., 12, 13, 14), but rather varies by 1, 2, or 3 bp from a ladder allele, then it will be designated as a microvariant of the smaller ladder allele that flanks the OL allele. For example, if an OL allele occurs between the allele size categories that represent alleles 12 and 13, and it is approximately 1 bp larger than the 12 ladder allele, the OL allele is designated a 12.1.

Alternatively, the off ladder allele can be designated by performing a visual interpretation of the allele using the *Show Bin* function in GMIDX.

8.3.5.3 If an OL allele does not fall within the size range of any locus-specific allelic ladder, it must be associated with one of the two allelic ladders between which it falls.

- a. If the OL allele is flanked by a locus with two peaks (i.e., heterozygous locus) and a locus with a single peak, the OL allele is assigned to the latter locus.
- b. If both loci between which an OL allele falls each display either a single allele or two alleles, the OL allele may be assigned to the locus closest in size to the OL allele.⁷

8.3.5.4 If an OL allele falls above the largest or below the smallest physically present ladder allele, the OL allele will be designated as either greater than (>) or less than (<) the respective ladder allele (e.g., >30).

8.3.5.5 Samples exhibiting OL alleles at the CODIS Core Loci must be re-worked for confirmation. At the discretion of a FDDU Examiner, a non-CODIS Core locus exhibiting an OL allele may be considered inconclusive and ineligible for entry into STACS and CODIS.

8.3.5.6 The allelic ladder may be periodically updated with additional “virtual” allele size

⁷ To facilitate the interpretation of OL alleles, the FDDU Examiner may consult a listing of such alleles recorded at http://www.cstl.nist.gov/div831/strbase/var_tab.htm.

categories. If this takes place during a sample's confirmation run, the GMIDX software may no longer identify the allele as off ladder. In these instances, it may be necessary for the FDDU Examiner to manually edit the allele in GMIDX to include the "OL" label. This designation is needed for STACS to complete the confirmation run and allow the sample to be uploaded to CODIS.

8.3.6 Tri-Allelic Patterns

Individuals can display three allelic peaks at a locus. Observed tri-allelic patterns are recorded at http://www.cstl.nist.gov/biotech/strbase/tri_tab.htm.

8.3.6.1 Samples exhibiting a tri-allelic pattern at the CODIS Core Loci should be re-worked for confirmation. If the tri-allelic pattern is confirmed, the allele values for the labeled peaks that meet the PIT will be imported into STACS and subsequently CODIS. At the discretion of a FDDU Examiner, a non-CODIS Core locus exhibiting a tri-allelic pattern may be considered inconclusive and ineligible for entry into STACS and CODIS.

8.3.6.2 A peak in a tri-allelic pattern may have a lower RFU value than the sister peaks at that locus, which may either fall below the PAT or be filtered out by GMIDX during the confirmation process.

8.3.6.2.1 At the discretion of a FDDU Examiner, if the third allele appears again during the confirmation process, the allele designation may be removed to allow the remaining peaks at that locus to be entered into STACS and subsequently CODIS.

8.3.6.2.2 If this third allele is \geq PIT, but filtered out by GMIDX during the confirmation run, the FDDU Examiner may manually add the allele label to the peak so that the verification can be completed in STACS.

8.3.7 Amelogenin

A specimen of male origin will exhibit both the X and Y amelogenin fragments, whereas a specimen of female origin will exhibit only the X fragment. However, primer binding site variants may occur at the amelogenin genetic locus that result in peak height ratios of significantly $< 60\%$ between the X and Y fragments. In these instances, the X peak may be filtered out or completely absent from the electropherogram, giving the appearance of a Y,Y result. In these instances the Y,Y result will be imported into STACS and subsequently uploaded to CODIS (Amelogenin results are uploaded to CODIS, but are not searched).

9 Calculations

9.1 Stutter

Stutter is most often observed as a minor PCR product peak. Stutter is expressed as a percentage relative to the source allelic peak.

$\% \text{ Stutter} = \text{Peak Height (stutter peak)} / \text{Peak Height (source allelic peak)} \times 100$

9.2 Non-Template Dependent Nucleotide Addition (-A)

Minus-A fragments are one base pair (bp) shorter (N-1) than the allelic fragment (N). Minus-A is expressed as a percentage relative to the allelic peak (i.e., % minus-A).

$\% \text{ Minus A} = \text{Peak Height (N-1)} / \text{Peak Height (N)} \times 100$

9.3 Peak Height Ratios (PHR)

For alleles at a heterozygote locus, the PHR is expressed as a percentage:

$\text{PHR (\%)} = \text{Peak Height (allele with lower RFU value)} / \text{Peak Height (allele with higher RFU value)} \times 100$

10 Limitations

The FDDU Examiner that performs the secondary analysis (technical review) must be different from the FDDU Examiner that performed the primary analysis (interpretation).

11 Sampling or Sample Selection

Not applicable.

12 Measurement Uncertainty

Not applicable.

13 Safety

Not applicable.

14 References

The procedures described here are derived from a variety of sources. Portions of the protocol come directly from some of the references cited below.

Quality Assurance Standards for DNA Databasing Laboratories, Current version.

DNA Procedures Manual.

FBI Laboratory Quality Assurance Manual.

National Research Council. The Evaluation of Forensic DNA Evidence. Washington, DC, National Academy Press (1996).

STACS DNA Inc. *Sample Tracking and Control System (STACS) User's Guide*.

Applied Biosystems. GeneMapper® ID-X Software Version 1.3 Getting Started Guide, Foster City, CA.

Applied Biosystems. AmpF/STR® Identifiler® PCR Amplification Kit User's Manual.

Applied Biosystems. AmpF/STR® Identifiler® Direct PCR Amplification Kit User Guide.

Rev. #	Issue Date	History
6	12/09/16	Added methods/procedures for processing samples with Globalfiler Express kit. Removed STACS specific interactions throughout. Simplified GMIDX interactions. Adjusted references as needed and eliminated redundant sections where possible. Added additional language for interpreting Tri-Allelic patterns. Defined PIT and PAT and replaced the RFU values throughout.
7	02/28/18	1 Updated scope 5.3.2 Updated Identifiler panel 6.7.5.2 Added notes about label editing exemptions 8.3.5.2 Added “physically present” within the “allelic” ladder 8.5.5.6 Added to address additional virtual bins 14 Updated reference to DNA Procedures Manual

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 02/27/2018

FDDU Chief

Date: 02/27/2018

QA Approval

Quality Manager

Date: 02/27/2018

FDDU

Procedures for Capillary Electrophoresis using the 3500xL

1 Scope

These procedures apply to DNA personnel that prepare daughter plates of samples amplified with the GlobalFiler® Express PCR Amplification Kit followed by the separation and detection by capillary electrophoresis (CE) with an Applied Biosystems (AB) 3500xL Series Genetic Analyzer in the Federal DNA Database Unit (FDDU).

2 Equipment/Materials/Reagents

Equipment/Materials

- General Laboratory Supplies (e.g., pipettes, tubes)
- Barcode printer with appropriately sized labels (2.0" x 0.5" or equivalent)
- Barcode Scanner, Hand-held (Symbol LS4000i, 4008i, LS4071 or equivalent)
- STACS (Sample Tracking and Control System) Software (STACS DNA Inc.), version 6.0 or higher
- Robotic Workstation (Tecan EVO 150/200)
 - Tecan EVOware Software, version 2.0 or higher (Tecan)
- 96-Well Sample (MicroAmp) Plates (Applied BioSystems or equivalent)
- 96-Well Plate Septa (Applied BioSystems or equivalent)
- Plate Sealer, microplate (Agilent Plate Loc or equivalent) with heat sealing plastic
- Thermal Cycler (Applied BioSystems GeneAmp® PCR System 9700 or ProFlex™ PCR System)
- Optical Compression Pad (Applied BioSystem or equivalent)
- 3500xL Genetic Analyzer (Applied BioSystems)
 - 3500 Series Data Collection Software, version 3.1 or higher (Applied BioSystems)
 - 96-Well Plate Base and Retainer (Applied BioSystems or equivalent)
 - 24-Capillary Array (3500xL), 36 cm (Applied BioSystems)

Reagents

- GlobalFiler® Express Allelic Ladder (Applied Biosystems)
- Hi-Di™ Formamide (Applied BioSystems or equivalent)
- GeneScan 600 LIZ® Size Standard v2.0 (Applied BioSystems)
- Anode Buffer Container (ABC), (Applied BioSystems)
- Cathode Buffer Container (CBC), (Applied BioSystems)
- Performance Optimized Polymer, AB 3500xL POP-4™, 384 sample pouch or 960 sample pouch (Applied BioSystems)
- Bleach, 3% (household or equivalent)
- Liquinox™ Critical Cleaning Liquid Detergent (Alconox or equivalent)
- DS-36 Matrix Standard Kit (Dye Set J6) (Applied BioSystems)
- Conditioning Reagent, (Applied BioSystems)

3 Standards and Controls

The following controls are included on each amplification plate and will be transferred for capillary electrophoresis. These controls will be interpreted according to the criteria in the applicable FDDU Procedure (i.e., FDDU 315).

GlobalFiler Express (GFE)
Negative (aka Combo)
Blood/Buccal Internal Standard (BIS)

4 Procedures

Refer to DNA Procedure Introduction (i.e., DNA QA 600) for applicable laboratory quality assurance and cleaning instructions.

When using a Robotic Workstation, ensure general instrument cleaning and maintenance is done prior to use, as needed. See the FDDU amplification procedure (i.e., FDDU 305) Appendix A for additional guidance.

4.1 Post Amplification Incubation

- 4.1.1 Load each 96-Well amplification plate into a thermal cycler and place an ABI Optical Compression Pad on each plate. Close the thermal cycler.
- 4.1.2 Select the appropriate method on the thermal cycler and ensure that the method displayed on the instrument screen matches the method outlined below:

HOLD 60°C for up to 20 minutes
HOLD 4°C Forever (∞)

- 4.1.3 Start the thermal cycler. Ensure that the reaction volume is 15 μ l and the ramp speed is 9600 on a 9700 or GeneAmpTMPCR System 9700 simulation mode on a ProFlex.
- 4.1.4 Ensure the *Thermal Cycler Bar Code* and the *Plate Bar Code* for each plate to be incubated has been scanned into STACS.
- 4.1.5 Indicate the result of the process in STACS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results.

4.2 Electrophoresis Plate Preparation (EPP) Daughter Plate Creation

- 4.2.1 Create the daughter plate in STACS.

4.2.2 Upon completion of the daughter plate creation, STACS prints out plate barcodes with a daughter plate designation. Place each barcode accordingly, on the EPP MicroAmp plate and support base.

4.2.3 Scan the barcodes affixed to both the EPP MicroAmp plate and support base. STACS verifies the scanned barcodes.

4.2.4 Repeat for each plate that is being processed at EPP.

4.3 GS-600 [LIZ] v2.0 Formamide Preparation

4.3.1 Prepare the GS-600 [LIZ] v2.0 formamide. The solution is prepared by combining Hi-Di formamide with GS-600 [LIZ] size standard v2.0 in a 19:1 ratio. Extra wells should be included in the calculation for overage.

GS-600 [LIZ] v2.0 Formamide (per 96-Well daughter plate)	
Hi-Di™ Formamide (9.5µl per reaction)	950 µl
GS-600 [LIZ] size standard v2.0 (0.5µl per reaction)	50 µl

4.3.2 Ensure the preparation has been recorded in the *Chemical Preparation* module of STACS.

4.3.3 Store the GS-600 [LIZ] v2.0 formamide solution refrigerated and use within the same day as it was prepared.

4.4 Electrophoresis Plate Preparation (EPP)

The EPP procedure can be performed either manually or by the robotic workstation.

4.4.1 Quick-spin the 96-Well Amplification plate(s) for approximately 30 seconds.

4.4.2 Within STACS, select the daughter plate(s) to be processed and select the appropriate scenario.

4.4.2.1 Additionally, for automated processing only:

- Scan the instrument barcode on the Tecan EVO Robotic Workstation.
- Ensure the Robot Maintenance Checks have been performed.
- Indicate whether each check passed.

4.4.3 Scan the barcode on each of the 96-Well Amplification plate(s), the EPP Daughter plate(s) and each reagent required for the selected scenario.

4.4.4 Select "Process" and proceed with the EPP procedure.

4.4.4.1 For manual processing, remove the seal cover from the 96-Well Amplification plate.

4.4.4.2 Additionally, for automated processing only:

- STACS launches the robotic software for the Tecan EVO Robotic Workstation
- If necessary, enter the appropriate user name and password at the robotic software log-in screen.
- Verify that the appropriate EPP script has been opened.
- Remove any plastic cover(s) on the 96-Well Amplification plate(s), if necessary.
- Ensure the selected 96-Well Amplification plate(s), the corresponding EPP Daughter plate(s) and the required reagents have been loaded on to the instrument.
 - Well A1 should be in the back right corner of the hotels.
- Ensure the instrument has been properly flushed and no air bubbles are visible in the tubing or syringes.
- Start the EPP script.
- Indicate the number of plates to be processed.

The following EPP procedure will be performed manually or by the Robotic Workstation:

4.4.5 Aliquot 10 µl of the GS-600 [LIZ] v2.0 formamide solution into each well of the EPP Daughter plate(s).

4.4.6 Add 1 µl of PCR product from the 96-Well Amplification plate(s) to its corresponding sample well in the EPP Daughter plate(s) and 1 µl of the appropriate allelic ladder to the designated well(s) in the EPP Daughter plate(s).

4.4.7 The Tecan EVO Robotic Workstation will re-seal the 96-Well amplification plate(s) with a plastic cover. For manually processed plate(s), heat seal the 96-Well Amplification plate(s) with a plastic cover.

4.4.8 Visually inspect the EPP Daughter Plate(s).

4.4.9 Indicate the result in STACS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results. If the plate(s) were processed on the Tecan EVO Robotic Workstation, indicate in STACS whether the bleach process was performed.

4.4.10 Ensure the EPP Daughter plate(s) are covered with septa.

4.4.11 Vortex (approximately 2 seconds) and quick-spin (approximately 30 seconds) the EPP Daughter Plate(s).

4.4.12 Return the 96-Well Amplification plate(s) to refrigerated storage in the post-amplification laboratory until data analysis has been completed, at which time the plate(s) can be discarded.

4.5 Electrophoresis Preparation Denature

- 4.5.1** Load the EPP Daughter Plate(s) into the thermal cycler(s). Close the thermal cycler.
- 4.5.2** Select the appropriate method on the thermal cycler and verify that the method displayed on the instrument screen matches the method outlined below:
 HOLD 95°C 3 minutes
 HOLD 4°C 3 minutes
 HOLD 4°C Forever (∞)
- 4.5.3** Start the thermal cycler. Ensure that the reaction volume is 11 μ l and the ramp speed is 9600 on a 9700 or GeneAMP™PCR System 9700 simulation mode on a ProFlex.
- 4.5.4** Ensure the *Thermal Cycler Bar Code* and *EPP Daughter Plate Bar Code* for each plate to be denatured has been scanned into STACS.
- 4.5.5** Indicate the result of the process in STACS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results.

4.6 Setting Up the 3500xL Genetic Analyzer (Sequencer)

NOTE: *If sequencer general maintenance is required, refer to Appendix A for guidance.*

- 4.6.1** Start the computer attached the 3500xL Genetic Analyzer (3500).
- 4.6.2** Ensure that the oven and all instrument doors are shut. Press the power button on the front of the analyzer to start the instrument. Ensure that the green status light is on before proceeding.
- 4.6.3** After the 3500 server monitor has fully initialized, log onto the workstation and then launch the 3500 Series Data Collection Software application.
- 4.6.4** Check consumable status in the dashboard. Replenish the consumables (e.g., POP-4, Anode Buffer Container [ABC], Cathode Buffer Container [CBC], or Array), if necessary. **Caution:** *To avoid electrical arcing, all surfaces of the containers must be clean and dry.*

Consumable		Frequency (whichever comes first)
POP-4 (24-cap)	960 sample pouch	14 days, 960 samples, or 50 injections
	384 sample pouch	14 days, 384 samples, or 20 injections
ABC and CBC	24-cap	14 days or 100 injections
Capillary Array	24-cap	As needed

- 4.6.5 The oven may be turned on and the temperature set in advance to shorten the interval between run activation and execution. It takes approximately 10 minutes to reach the target temperature.
- 4.6.6 Ensure the chemicals/reagents/array required for the Genetic Analyzer have been defined and/or verified in STACS using the *Sequencer Configuration* module.
- 4.6.7 If necessary, ensure any changes to chemicals/reagents/array performed on the Genetic Analyzer have been recorded in STACS using the *Instrument Maintenance* module.

4.7 Post PCR and Sample Sheet Creation

In the *Post PCR* module, the type of Genetic Analyzer (*Sequencer*) to be used for capillary electrophoresis is selected, the reagents assigned to the sequencer are recorded and a sample sheet(s) generated.

- 4.7.1 Within STACS, scan the *Sequencer Bar Code* of the selected instrument that will be utilized to perform capillary electrophoresis.
- 4.7.2 Scan the barcode on the Electrophoresis (EPP) daughter plate(s) to be processed.
- 4.7.3 Specify the *Destination Directory* for the sample sheet(s) in STACS.
- 4.7.4 Create the sample sheets. STACS creates a sample sheet file with the same name as its corresponding EPP daughter plate barcode.

NOTE: If multiple injections of any FDDU samples and/or controls are required, the user may add the injections during the creation of the sample sheet or later in the Data Collection software of the sequencer.

- 4.7.5 Indicate the result of the process in STACS as successful, failed or aborted. Comments and observations must be entered for plates with process failed results.

4.8 Initiating Capillary Electrophoresis on a 3500xL Genetic Analyzer

- 4.8.1 Define the Plate Properties for the EPP daughter Plate to be run.
 - Record sample plate (barcode) information if needed.
 - Choose *Workflow - Assign Plate Contents* from the navigation panel.
 - Select *Import* and browse to the STACS generated sample sheet file(s).
 - Select *Open* to import the Plate into the 3500 series Data Collection Software.
- 4.8.2 Review the plate contents for each imported plate in the Table View tab and bottom of the Assign Plate Contents screen
 - Ensure that the *Sample Name* column contains expected sample barcodes.
 - Ensure the *Sample Type* for Ladders and Controls fields are complete.

- Ensure the assigned Assay(s), *Results Group* and File Name Convention fields are correct for the selected instrument and sample/plate type.
- Use the edit button and/or pull down menus to make any necessary changes to the spreadsheet.
- Additional Assays may be created (saved in the Library) from the Workflow - Assign Plate Contents screen that include multiple injections of all samples and/or controls the assay is applied to. Choose "Add From Library".
- Save the plate and any changes to the Library.

Repeat above steps to add a 2nd plate to be run on the Genetic Analyzer, if necessary.

NOTE: The parameters defined in an *Instrument Protocol (dye set and run configuration)* may vary between AB 3500xL Genetic Analyzers. The *Instrument Protocol(s)* for each instrument are available for reference.

4.8.3 Load Plates for the Run

- Ensure each AB 3500xL plate assembly has been securely prepared with the EPP Daughter Plate and the plate assemblies are properly seated in the autosampler with the instrument door closed.
- Click *Link Plate for Run* or *Load Plates for Run* in the navigation panel to assign the plate(s) from the plate library, and specify the position of the plate(s) in the autosampler (A and/or B).

4.8.4 Confirm that the linked plate(s) are in the correct position of the autosampler. Click *Create Injection List* to review the injection list and/or make any changes. Choose Preview Run on the left navigation panel before starting the run.

4.8.5 If necessary, to add injections of any FDDU samples and/or controls not added at Assign Plate Contents to the injection list:

- Select the sample(s) to be injected multiple times by highlighting the appropriate row. Click *Duplicate* to add injection(s) immediately following the original injection or after all injections have finished.
- The same Assay, Results Group and File Naming Convention parameters for the FDDU samples and/or controls in the original injection will be used. Select "OK" to save the plate.

4.8.6 Ensure there are no bubbles in the polymer delivery pump. The Data Collection Wizard for bubble removal may be run, if necessary.

4.8.7 Start the Run.

NOTE: The electrophoresis run can be monitored by selecting Monitor Run from the navigation panel.

5 Sampling

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

The appropriate processing methods are selected for a plate based on the sample type added to the plate and the amplification kit to be used. Based on internal studies, only the combinations of processes listed below are approved for use.

Sample Type	Amp Kit	EPP	Genetic Analyzer
Blood [Non-FTA] and Buccal [FTA and Non-FTA]	GlobalFiler Express (GFE)	Manual or Automated using a Tecan EVO Robotic Workstation	AB 3500xL

9 Safety

9.1 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.2 Procedural Specific Chemical Hazard:

- Formamide is a teratogen. Avoid inhalation, skin contact, or ingestion. Use nitrile gloves when handling. Dispose of unused portions in appropriate hazardous waste containers. Pregnant women must not handle formamide.
- Performance Optimized Polymer is caustic. Avoid inhalation, skin contact, or ingestion. Use gloves when handling. Dispose of unused portions in appropriate hazardous waste containers.

10 References

FBI Laboratory Quality Assurance Manual.

FBI Laboratory Safety Manual.

DNA Procedures Manual.

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Rev #	Issue Date	History
0	12/09/16	New document for the implementation of Globalfiler Express and the 3500xL.
1	12/02/19	1: Updated Scope to apply to DNA personnel 2, 4.1.3, 4.5.3, 10: Added ProFlex and parameters 4.8: Adjustments to steps for initiating the 3500 run Appendix A: I.2: Added for polymer to come to room temp; II.5.f: Clarified 2 pixel criteria; IV,V,&VI: Clarified that only 007 samples are used for LIZ calculations.

Approval

Redacted - Signatures on File

FDDU Unit Chief

Date: 11/29/2019

DNA Technical Leader

Date: 11/29/2019

Appendix A: CE Maintenance and Performance Verification Procedures

General maintenance and performance verification procedures are performed in accordance with the LOM practice and DNA procedure for equipment calibration and maintenance. General maintenance is generally performed at the intervals listed below. Performance verification of the genetic analyzer instruments will be conducted at the minimum frequency described in the DNA procedure for equipment calibration and maintenance.

3500xL General Maintenance	Recommended Interval
Replace Polymer	14 days (or as required in procedure)
Replace Buffer (Anode and Cathode)	14 days (or as required in procedure)
Install New Array	As needed
Flush pump chamber and channels	Weekly
Flush pump trap	Monthly
Database Cleanup	Weekly
Data Backup	Weekly
Disk Defragmentation	As needed
Spatial Calibration	With array change or as needed
Spectral Calibration	With array change or as needed
GS600 LIZ Sensitivity Evaluation	Semiannually or after optical adjustment

I. General Maintenance of the Applied Biosystems 3500xL

1. Flush the pump chamber and channels
 - a. From the Maintenance Wizards screen, click **Wash Pump and Channels**.
 - b. Follow the prompts in the Wash Wizard window. The Wash Pump and Channels wizard takes approximately 40 minutes to complete.
2. Replace polymer (POP-4) pouch. Allow refrigerated polymer to equilibrate to room temperature prior to first use. Follow prompts in Maintenance Wizards screen, select Replenish Polymer.
3. Replace Anode Buffer Container (ABC)
 - a. Allow refrigerated ABC to equilibrate to room temperature prior to first use. Do not remove seal.
 - b. Verify that buffer level is at or above the fill line and check that seal is intact
 - c. Invert the ABC, then tilt slightly to make sure most of the buffer is in the larger side of the container. There should be less than 1mL of the buffer remaining in the smaller side of container.
 - d. Verify that the buffer is at the fill line.
 - e. Peel off the seal at the top of the ABC. Place the ABC into the Anode end of the instrument, below the pump.
 - f. Close the instrument door and click **Refresh** on the Dashboard to update status after changing the ABC.
4. Replace the Cathode Buffer Container (CBC)
 - a. Allow refrigerated CBC to equilibrate to ambient temperature prior to first use.
 - b. Wipe away condensation on the CBC exterior with lint-free lab cloth.
 - c. Verify that buffer level is at or above the fill line and check that seal is intact.

- d. Tilt the CBC back and forth gently to ensure that the buffer is distributed evenly across the container.
 - e. Verify that the buffer is at or above the fill line.
 - f. Place the container on a flat surface and peel off the seal.
 - g. Wipe off any excess buffer on top of the CBC and ensure that the top of the container is dry.
 - h. Place the appropriate septa on both sides of the CBC.
 - i. Click the **Tray** button on the front panel to move the autosampler to the front position.
 - j. Install the CBC on the autosampler. The CBC will click into the autosampler as the tabs are snapped into place.
 - k. Close the instrument doors. Click **Refresh** from the Dashboard to update status after changing the CBC.
5. Maintenance of the storage databases used by the Data Collection software.
 - a. Open the appropriate results group folder and create a new backup folder using the naming convention, CE#XX_Backup_MMDDYY.
 - b. Move all plate folders into the newly created backup folder and then copy it to the appropriate CE Backup folder on the network. (Example: \\FS1\CE Backup)
6. Delete plate records from the database
 - a. From the navigation pane, select Library – Plates and choose Manage – Purge.
 - b. Allow the software the appropriate amount of time to delete the associated records and close the dialog box once complete.
7. Defragment the data storage (E:) hard drive using disk management in Windows system tools.

II. Array Change and Spatial Calibration

The capillary array will be changed as needed. The determination to change the array will be based upon a review of the quality of the data generated by the instrument.

1. From the Maintenance Wizards screen, click **Install Capillary Array**
2. Install the array as instructed by the wizard being careful not to leave fingerprints on the detection window.
 - a. Ensure the proper type (24-cap) and length (36 cm) is entered in the array information fields.
3. In the final step of the wizard you can choose to fill the array with polymer or click “finish” if the array will be filled during the spatial calibration.

A spatial calibration must be performed whenever a new array is installed or every time the detection cell window is opened.

1. Select **Maintenance** in the navigation pane then select **Spatial Calibration**
2. Select **Fill** to fill the array with polymer before starting the calibration
3. Select **Perform QC Checks**
4. Click **Start Calibration** button to initiate the spatial calibration.
5. Select **Accept Results** to accept the spatial calibration if the following criteria are met:
 - a. Peaks of the spatial calibration are approximately the same height.
 - b. One marker(a cross) appears at the top (apex) of each peak in the profile.
 - c. No irregular peaks are contained in the profile

- d. RFU (relative fluorescence unit) values for the peaks are greater than 3000 for a 24-cap 3500xL array.
- e. Uniformity or peak height similarity values are 0.2
- f. The values for the Capillary spacing are ≤ 2 pixels

III. Spectral Calibration

A spectral calibration must be performed whenever a new array is installed or as needed (e.g., decrease in spectral separation, new dye set, or optical adjustment). A spectral plate may be injected several times within a 24 hour period; a fresh spectral plate should be used for each instrument being calibrated.

1. Combine 294 μ l of formamide with 6 μ l of DS-36 Matrix Standard for the J6 dye set.
2. Dispense 10 μ l of solution into the first three columns (A1-H1, A2-H2, and A3-H3).
3. Spin down and denature plate on thermal cycler then place on instrument.
4. Access the Spectral Calibration screen: Select **Maintenance**, then select **Spectral Calibration** in the Navigation pane.
5. Select number of wells on the plate (e.g., 96 well plate) and specify plate position on instrument.
6. Select the chemistry standard and dye set for the calibration plate.
7. Select **Allow Borrowing**.
8. Click Start Run.

Pass Criteria: The data collection software indicates the pass/fail status of each capillary. The spectral calibration is acceptable if the following criteria are met, and there is proper separation between the color channels.

1. All capillaries have to meet the spectral Quality Value and Condition Number limits.
2. The passing Quality Value for J6 Dye Set is a minimum of 0.95.
3. The passing Condition Number value is a maximum of 8.0 for J6 Dye Set.
4. ≤ 3 adjacent-capillary borrowing events allowed

The software gives a pass/fail status to each capillary. The user must evaluate the spectral profile traces and **Accept Results** or **Reject Results**.

IV. Performance Verification

Performance verification (PV) of the genetic analyzer instruments should be conducted at the minimum frequency described in the DNA procedure for equipment calibration and maintenance.

1. Using 007 amplified with the Globalfiler Express amplification kit for 26 cycles.
2. Prepare a stock solution in a 10:1 ratio of GS600LIZ/Formamide to 007 amplicon.
3. Add 11 μ l of the stock solution to 21 wells in the first 3 columns of a plate.
4. Add 10 μ l of GS600LIZ/Formamide and 1 μ l of Globalfiler Express ladder to the remaining 3 wells in the first 3 columns of the plate.
5. Inject the samples two times at the instrument's current injection setting

Pass Criteria: A genetic analyzer will be deemed suitable for FDDU analysis if:

1. All peak heights of all alleles in the 007 positive control are greater than 175 RFU when analyzing the data with normalization.
2. Correct typing results obtained for 007.
3. The average peak heights of the 007 samples for the 11 GS600 LIZ peaks used for normalization is between 1675 and 5025 RFU when analyzing the data without normalization.

The following are the 11 peaks used for normalization:

200	220	240	260	280	300	314	320	340	360	400
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V. Quality Control of GS600 LIZ v2 Internal Size Standard

1. Prepare a plate using the instructions in section IV using the new lot of GS600 LIZ.
2. Run the plate on an appropriate 3500xL instrument.
3. Analyze the data without normalization.
4. The sensitivity of the new lot will be accepted if the average peak heights of the 007 samples for the 11 GS600 LIZ peaks used for normalization (listed above) is between 1675 and 5025 RFU.
5. If the lot of GS600 does not meet sensitivity expectations, the assessment will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

VI. Sensitivity Evaluation using GS600 LIZ v2

1. Prepare a plate(s) using the instructions in section IV. A single plate or multiple plates prepared with the stock solution may be used to evaluate multiple 3500xL instruments. Run the plate(s) on the appropriate 3500xL instrument(s). A plate prepared for or data generated from section IV may also be used for this evaluation.
2. Analyze the data without normalization.
3. The sensitivity of each instrument will be accepted if the average peak heights of the 007 samples for the 11 GS600 LIZ peaks used for normalization (listed above) is between 1675 and 5025 RFU.
4. If one or more instruments do not meet sensitivity expectations, the evaluation for that instrument will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

DNA

Procedures for Preparation and Extraction of Calcified Tissue Samples

1 Scope

These procedures describe the process for chemical digestion and purification of deoxyribonucleic acid (DNA) from calcified tissue (i.e., teeth and bones) for nuclear and/or mitochondrial DNA testing.

2 Equipment/Materials/Reagents

Equipment/Materials

- General laboratory supplies
- Centrifuge (Sorvall™ Legend™ XT, Heraeus™ Megafuge™ 16, or equivalent)
 - 15ml conical tube inserts
- Centrifuge (Eppendorf Minispin Plus, Hermle MR-2, or equivalent)
- Freezer Mill (SPEX® 6750 or 6770, or equivalent)
 - Pulverization cylinder, end plugs, impactor bar
 - Extractor
- Hood Prefilter (Air Clean ACF PRE or equivalent)
- Isopropanol Wipes
- Incubator (Lab-Line Imperial III or equivalent) with nutator
- Rotary tool (Dremel® or equivalent)
 - Rotary tool accessories
 - Sanding disc(s) or barrel(s)
 - Cutting disc(s)
- Sonicator (Fisher Scientific FS-20 or equivalent) or nutator, if needed
- Vivacon® 2 ETO concentrators, 50kDa/50,000 MWCO
- Qiagen® MinElute® spin columns

Reagents

- DNA-OFF™ or 10% Bleach Solution
- Liquid Nitrogen
- Demineralization/Extraction Buffer (Demin Buffer)
- 20mg/mL Proteinase K (ProK)
- Terg-a-zyne® (or equivalent)
- 25:24:1 Phenol/Chloroform/Isoamyl Alcohol (PCIA)
- Qiagen® Buffer PB
- Qiagen® Buffer PE
- Reagent Grade Water

3 Standards and Controls

At least one extraction control (i.e., reagent blank) must be processed in parallel with each extraction sample.

For evaluation of the extraction controls, refer to the appropriate interpretation procedure of the *DNA Procedures Manual*.

4 Sample Selection

If soft tissue, tooth pulp or bone marrow is present, DNA may be extracted using the appropriate DNA procedure for the extraction of DNA from body fluids and tissues. If several teeth are available for analysis, non-restored teeth are preferred over restored teeth, and molars are preferred over non-molars. If several bones are available, order of preference for bone selection for DNA extraction is generally as follows:

- 1) Long bone
- 2) Rib (mid-section)
- 3) Other (determined by Examiner)

5 Procedures

Refer to DNA Procedure Introduction (DNA QA 600) for applicable laboratory quality assurance and cleaning instructions. For previously powderized samples, proceed to sample processing.

5.1 Equipment Preparation

Supplies needed for sample preparation may include:

- Freezer mill sample vial assembly (cylinder, end plugs, impactor bar)
- Rotary tool and accessories (cutting disc(s) and sanding disc(s) or barrel(s))
- Weigh paper or weigh boats
- 15 mL conical tubes (typically 7) and 15 mL tube rack
- Ruler
- Tweezers (optional)
- Screwdriver (optional)

5.1.1	Agitate cylinder, end plugs, and impactor bar in DNA-OFF or 10% bleach for at least 20 minutes. Rinse at least 3 times with distilled water.	
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5.1.2	Agitate in reagent grade water for at least 20 minutes.	
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Wipe cylinder, end plugs, and impactor bar with isopropanol prior to use.

5.2 Sample Preparation

Specimen may be photographed with label and ruler(s) before and after processing.

The following steps will be performed wearing a disposable lab coat.

5.2.1	In an appropriate hood, use sanding disc or barrel to sand outer surface of sample around area to be excised. Clean sanded area with isopropanol.	
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5.2.2	Use cutting disc to remove tooth root(s) or ~2 cm x 2 cm section of bone. Clean the cutting(s) with isopropanol.	
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When possible, avoid any previous bone cuttings or tooth restorations that may be present.

Sand interior of any excised portion, if necessary.

If evidence will be consumed, clean by rinsing, incubating, or sonicating (~15-20 minutes) with 5% Terg-a-zyme. Then follow by rinsing, incubating, or sonicating (~15-20 minutes) with reagent grade water. Allow sample to dry before proceeding.

Excised portion(s) may be stored at 4°C or colder prior to pulverization.

If marrow or pulp is present, remove with tweezers, place in a UV'd 1.5 mL tube, and process according to the appropriate procedure for the extraction of DNA from body fluids and tissues.

5.2.3	Place excised portion of bone or tooth into assembled sample vial.	
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5.2.4	Ensure liquid nitrogen is to the fill line then pulverize the sample using a freezer mill.	
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Run 6750 freezer mill as follows: T1: 5.0 T2: 2.0 T3: 0.1 Rate: 15

Run 6770 freezer mill as follows: (Touch screen to activate program screen.)

Cycles: 1 Precool: 0 min. Run Time: 5 min. Cool Time: 2 min. Rate: 15 CPS

5.2.5	When grinding is complete, open lid and remove cylinder.	
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If sample is not sufficiently pulverized, reinsert for additional grinding. Additional liquid nitrogen may be necessary.

5.2.6	Transfer powderized sample to appropriately labeled tube(s): Pour ~0.2 g of powder into a 15 mL conical tube (approximately to the 0.2 mL line on the tube). Create a total of 3 tubes of ~0.2 g of powder, if enough sample is available.	
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Store remaining powder, if applicable, in a conical tube at 4°C or colder. Case notes should indicate the approximate amount of powder remaining.

Powder sample(s) may be stored at 4°C or colder prior to initiating extraction process by adding Demin Buffer.

The following may be done at the most convenient point after use:

- Clean rotary tool with bleach.
- Clean rotary tool accessories with Terg-a-zyme solution.
- If reusing end pieces, bar, and/or cylinder, clean with Terg-a-zyme solution, then repeat initial cleaning.
- Clean hood with bleach and replace hood prefilter.

Disposable lab coats used for sample preparation should not be worn in laboratory space used for extraction and amplification set-up procedures.

5.3 Powderized Sample Processing

Supplies needed for sample powder processing may include:

- Demin Buffer
- ProK (Do not UV)
- p200, p1000 pipettes

5.3.1	<p>Add 3 mL Demin Buffer and 200 µL ProK to sample tube(s). Close tube(s) and vortex gently to suspend powder.</p> <p>Add 3 mL Demin Buffer and 200 µL ProK to RB tube(s). (The number of RB tubes will be equal to or greater than the number of sample tubes).</p> <p>Close tube(s) and vortex gently.</p> <p>Incubate tubes at 56°C with agitation for 4 to 48 hours.</p>	
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Sample(s) should be incubated until completely digested; however, samples that are not completely digested may proceed to purification steps.

5.4 PCIA/Vivacon Purification

Supplies needed for PCIA/Vivacon purification may include:

- 15 mL tubes (typically 6) and tube rack
- PCIA (Do not UV)
- Vivacon concentrators (typically 6) and tube rack
- Reagent grade water
- p20, p200, p1000 pipettes

The inside of the centrifuge should be wiped with isopropanol before use.

PCIA and all consumables that come into contact with PCIA (i.e., tips, tubes) must be disposed of in an appropriate waste container.

5.4.1	In a fume hood, add 3 mL PCIA to each tube. Vortex briefly and centrifuge at ~4200 x g for 8 minutes.	
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If upper aqueous layer is still cloudy, additional centrifuge time may be required.

5.4.2	Transfer aqueous layer from each tube to a new, labeled 15 mL conical tube. Avoid transferring the interface. In a fume hood, add 3 mL PCIA to each tube. Vortex briefly and centrifuge at ~4200 x g for 8 minutes.	
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If upper aqueous layer is still cloudy, additional centrifuge time may be required.

5.4.3	Add 700 µL reagent grade water to each labeled Vivacon concentrator. Transfer ~1300 µL of the aqueous layer from each tube to the corresponding Vivacon. Avoid transferring the interface. Gently pipette mix. Centrifuge for 30 minutes.	
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For centrifuging the Vivacon concentrators:

- * When using a Sorvall™ Legend™ XT with fixed rotor (or equivalent), centrifuge at ~5860 x g.
- * When using a Heraeus™ Megafuge™ 16 with swinging-bucket rotor (or equivalent), centrifuge at ~5000 x g.

If volume is not low enough to add remainder of aqueous layer, centrifuge an additional 5-15 minutes or add reagent grade water, gently pipette mix, and centrifuge 5-15 minutes.

5.4.4	Discard waste. Add remainder of aqueous layer and/or reagent grade water to each corresponding Vivacon and bring volume to ~2 mL. Gently pipette mix. Centrifuge for 30 minutes or until volumes are ~700 µL or less (slightly above or anywhere below the “50K” on the Vivacon).	
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If volume in the Vivacons is not $\leq 700 \mu\text{L}$, centrifuge an additional 5-15 minutes or add reagent grade water, gently pipette mix, and centrifuge 5-30 minutes. If the volume in only some/one of the Vivacons is slightly greater than $700 \mu\text{L}$, those may be independently centrifuged an additional 5-15 minutes (use a balance if needed).

5.4.5	Discard waste. Add reagent grade water to each Vivacon to bring volume to $\sim 2 \text{ mL}$. Gently pipette mix. Centrifuge in 15 minute intervals until volume(s) $\leq 100 \mu\text{L}$.	
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If needed, add reagent grade water to Vivacon(s), gently pipette mix, and then centrifuge an additional 5-15 minutes. If the volume in only some/one of the Vivacons is slightly greater than $100 \mu\text{L}$, those may be independently centrifuged an additional 5-15 minutes (use a balance if needed).

5.4.6	Discard waste. Add reagent grade water to bring volume to $\sim 100 \mu\text{L}$ if necessary. Invert Vivacons into labeled recovery caps. Centrifuge at $2500 \times g$ for 2 minutes.	
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Wipe centrifuge with isopropanol and clean centrifuge inserts with 10% bleach and rinse with water after procedure.

5.5 MinElute Purification

Supplies needed for MinElute purification may include:

- Buffer PB
- Buffer PE
- MinElute columns in collection tubes (at least 2)
- Reagent grade water
- 1.5 mL or 2 mL tubes (at least 4)
- Tube rack
- p200, p1000 pipettes

****NOTE: MinElute columns must be at room temperature prior to use.** (Generally takes about 30 minutes.)

5.5.1	Add 500 μ L Buffer PB to each MinElute column. Transfer ~100 μ L from one sample Vivacon recovery cap to the sample MinElute column. Gently pipette mix. Transfer ~100 μ L from one RB Vivacon recovery cap to the RB MinElute column. Gently pipette mix. Centrifuge both at ~13000 x g for 1 minute. Discard waste and return column to collection tube. If applicable, repeat for each remaining sample(s) and RB(s) using the same corresponding MinElute column.	
5.5.2	Add 750 μ L Buffer PE to each MinElute column. Centrifuge at ~13000 x g for 1 minute. Discard waste and return column to collection tube.	
5.5.3	Centrifuge at ~13000 x g for 1 minute. Transfer columns to new, labeled, 1.5 mL or 2 mL tubes. Discard waste and collection tubes.	
5.5.4	Add 25 μ L reagent grade water to each column. Centrifuge at ~13000 x g for 1 minute.	
5.5.5	Add another 25 μ L of reagent grade water to each column. Centrifuge at ~13000 x g for 1 minute. Transfer RB(s) and sample elution to appropriate tube for future processing.	

If applicable, refer to the appropriate DNA procedure for concentrating extracted samples.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

The quantity and quality of the DNA present within any biological material ultimately determines if a DNA extraction is successful.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Procedural Specific Chemical Hazards:

- Solutions of Proteinase K can be irritating to mucous membranes. Use eye protection when handling.
- PCIA is an irritant and is toxic. Its use will be confined to a chemical fume hood whenever possible.
- Liquid nitrogen can be hazardous. Use appropriate PPE when handling.
- Buffer PB spills should not be directly cleaned with bleach as a combination of the two can form highly reactive compounds. Spills should be absorbed prior to cleaning.

10 References

DNA Procedures Manual

FBI Laboratory Safety Manual

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Rev. #	Issue Date	History
0	09/24/15	Original document issued. Combination and incorporation of procedural modifications of the Procedures for Extraction of DNA from Skeletal Remains (DNA 223-2) and the Calcified Tissue Extraction portion from Mitochondrial DNA Laboratory Protocol, Revision 1.

Approval

 Redacted - Signatures on File

DNA Casework Unit

Procedures for the Extraction of DNA from Hair and Keratinized Tissue

1 Scope

These procedures describe the process for chemical digestion and purification of deoxyribonucleic acid (DNA) from hair or keratinized tissue (i.e., fingernails).

2 Equipment/Materials/Reagents

Equipment/Materials

- General laboratory supplies (e.g., tubes, pipettes, vortex, forceps)
- Magnetic stand
- Stereomicroscope
- Sonicator
- Micro tissue grinder (aka mortar and pestle), if needed

Reagents

- Xylene and/or xylene substitute, if needed
- Terg-a-zyme, powder or 5% solution
- Ethanol (EtOH), absolute
- Qiagen® Buffer ATL
- Dithiothreitol (DTT), 5M solution
- Proteinase K (ProK), 20mg/mL
- Qiagen® Buffer AL
- PrepFiler® Forensic DNA Extraction Kit
 - PrepFiler® Magnetic Particles
 - PrepFiler® Wash Buffers A and B
 - PrepFiler® Elution Buffer (or TE⁻⁴ Buffer)
- Isopropanol, 70% Water, reagent grade or equivalent
- Sulfuric acid, 4 N, if needed

3 Standards and Controls

At least one extraction control (i.e., reagent blank [RB]) must be processed in parallel with each extraction batch.

For evaluation of the extraction controls, refer to the appropriate interpretation procedure of the *DNA Procedures Manual*.

4 Procedures

Refer to the DNA Procedures Introduction (DNA QA 600) for applicable general precautions and cleaning instructions.

Supplies typically needed for 1 sample and 1 RB (adjust for batches):

EtOH (~1 mL)	Isopropanol (~400 µL)	<u>Do NOT UV</u>
Water (~10mL)	Wash Buffer A (~2 mL)	Magnetic beads
5M DTT (~15 µL)	Wash Buffer B (~1 mL)	ProK
Buffer ATL (~1 mL)	Elution Buffer (~150 µL)	
Buffer AL (~1 mL)		

- 4 - 1.5 mL tubes (sample, RB, final sample extract, final RB extract)
- Ruler, magnetic stand, forceps, scissors, scalpel, tube rack
- p20, p200, p2000 pipettes

4.1 Sample Collection

Except as noted, the following steps are performed in a hood.

A description of the collected sample will be recorded in the notes.

4.1.1 Hair

Any step involving manipulations of difficult hairs may occur outside of hood with aid of stereomicroscope. Reverse action forceps may aid in grasping a hair. **The hood air flow must be off while transferring/handling hair samples.**

4.1.1.1	View hair under stereomicroscope for presence of root tissue or adherent material.	
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If sheath material is present, consult an Examiner.

4.1.1.2	Measure hair and record length. Remove ~ 2 cm of hair from root end and place in a tube.	
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The sample may be moistened with water to minimize the effects of static.

4.1.2 Fingernail Clipping

4.1.2.1	Place ~ 3 mm x 3 mm of a fingernail in a tube.	
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4.2 Sample Cleaning Methods

Each wash procedure may be performed additional times using fresh cleaning solution. The cleaning method(s) used will be recorded in the notes.

Pulse spin, as necessary, throughout procedure to force sample to bottom of tube. A sample may remain in the same tube for each cleaning procedure with the removal of the cleaning liquid(s). In instances with smaller samples, smaller rinse tubes and/or less liquid may be used.

4.2.1 Xylene Wash (Optional)

4.2.1.1	In chemical fume hood, add enough xylene to cover sample. Sonicate at least 20 minutes in chemical fume hood.	
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Alternatively, xylene may be added to the collection tube prior to the addition of sample.

4.2.1.2	Remove xylene and appropriately discard waste. Add enough reagent grade water to cover sample and mix.	
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As an alternative to the xylene removal, sample may be transferred from xylene tube to a separate water tube with use of tweezers.

4.2.2 Xylene Substitute Wash (Optional)

4.2.2.1	Transfer sample to tube containing enough UV-treated xylene substitute to cover sample. Sonicate at least 20 minutes.	
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4.2.3 Terg-a-zyne Wash

To make 5% Terg-a-zyne solution, add 0.5 g Terg-a-zyne to 10 mL water.

4.2.3.1	Transfer sample to tube containing enough 5% Terg-a-zyne solution to cover sample. Sonicate at least 20 minutes.	
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4.2.3.2	Transfer sample to tube containing enough EtOH to cover sample and mix to rinse.	
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4.2.3.3	Transfer sample to tube containing enough water to cover sample and mix to rinse.	
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4.3 Digestion

4.3.1	Enter appropriate barcodes and prepare Digestion Buffer.	
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To make Digestion Buffer, add 13.2 μ L 5M DTT to 1 mL Buffer ATL.

4.3.2	Add 300 μ L of Digestion Buffer and 20 μ L ProK to each sample and RB tube. Ensure samples are submerged. <i>Samples may be cut into pieces to ensure full immersion.</i> Vortex and incubate tubes at 56°C at 900 rpm for a minimum of ~30 minutes, until the sample is fully digested, or overnight (O/N).	
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Sample is generally transferred from the last cleaning tube to a new tube containing the digestion buffer; however, the sample may remain in the same tube with the removal of the water rinse prior to the addition of the digestion buffer.

NOTE: For keratinized tissues, a minimum incubation time of 2 hrs is recommended.

If full digestion does not occur after a minimum of 2 hrs, a partially-digested hair sample (and RB) may undergo the grinding process at the end of this procedure.

4.3.3	Pulse spin. Add 300 μ L Buffer AL. Vortex tubes and incubate at 70°C at 900 rpm for 10 minutes.	
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4.3.4	Pulse spin and allow to come to room temperature (~5 minutes).	
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4.4 PrepFiler® DNA Extraction Kit Purification

Prior to addition, vortex PrepFiler® Magnetic Particles tube for 5 seconds until no visible pellet remains in bottom of tube. Pulse spin. *If processing multiple samples, vortex every ~5 minutes.*

4.4.1	Add 15 μ L of Magnetic Particles. Vortex at low speed for 10 seconds. Pulse spin.	
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4.4.2	Add 180 μ L of isopropanol. Vortex at low speed for 5 seconds. Mix at room temperature at 1,000 rpm for 10 minutes in shaker.	
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4.4.3	Vortex at high speed for 10 seconds. Pulse spin.	
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4.4.4	Place tubes in magnetic stand. Wait until size of pellet on back of tubes stops increasing (~3 minutes). With tubes in magnetic stand, pipette off and discard all liquid without disturbing pellet.	
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4.4.5	Add 600 µL Wash Buffer A. Vortex at high speed until there is no visible pellet on side of tube (~5 seconds). Pulse spin.	
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It is acceptable to have visible aggregates in solution or on side of tube below meniscus.

4.4.6	Place tubes in magnetic stand for ~60 seconds. With tubes in magnetic stand, pipette off and discard all liquid without disturbing pellet.	
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4.4.7	Add 300 µL Wash Buffer A. Vortex at high speed until there is no visible pellet on side of tube (~5 seconds). Pulse spin.	
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It is acceptable to have visible aggregates in solution or on side of tube below meniscus.

4.4.8	Place tubes in magnetic stand for ~60 seconds. With tubes in magnetic stand, pipette off and discard all liquid without disturbing pellet.	
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4.4.9	Add 300 µL Wash Buffer B. Vortex at high speed until there is no visible pellet on side of tube (~5 seconds). Pulse spin.	
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It is acceptable to have visible aggregates in solution or on side of tube below meniscus.

4.4.10	Place tubes in magnetic stand for ~60 seconds. With tubes in magnetic stand, pipette off and discard all liquid without disturbing pellet. With tubes remaining in magnetic stand, open and air-dry in hood with blower for ~8 minutes.	
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DO NOT OVERDRY.

If the room temperature is >25°C, reduce the drying time to 5 minutes.

4.4.11	Add 65µL of Elution Buffer or TE ⁻⁴ . Vortex at high speed until there is no visible pellet on side of tube (~5 seconds). Pulse spin.	
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If an alternate volume is used for elution, record the volume in the case notes.

4.4.12	Incubate at 70°C and 900 rpm for 5 minutes. Vortex at high speed until there is no visible pellet on side of tube (~2 seconds). Pulse spin.	
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4.4.13	Place tubes in magnetic stand. Wait until size of pellet on back of tubes stops increasing (~2 minutes.). Transfer liquid into final extract tube without disturbing pellet.	
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If an extract is discolored, spin 10,000 X g for 7 minutes and transfer supernatant to new tube.

4.5 Grinding (If necessary)

If necessary, the following procedure may be utilized if full digestion has not occurred after a minimum of 2 hrs.

4.5.1	Moisten swab with 5% Terg-a-zyne solution. <i>Terg-a-zyne may be warmed prior to use.</i> Scrub mortar and pestle with swab. Simulate grinding. Rinse with water. Repeat two times.	
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4.5.2	In chemical fume hood, add 300uL 4 N sulfuric acid to mortar and simulate grinding. Soak mortar and pestle in 4 N sulfuric acid for 20 minutes.	
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4.5.3	Rinse the mortar and pestle with water. Pulse spin the pestle. Remove remaining water and crosslink.	
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4.5.4	Transfer 200 µl of solution from the RB tube to mortar and simulate grinding. Remove pestle from mortar. Transfer liquid back to RB tube.	
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4.5.5	Transfer 200 µl of solution and the undigested sample fragments to mortar and grind until fragments are no longer visible. Remove pestle from mortar. Transfer liquid back to sample tube.	
4.5.6	Add 20µL ProK and 4µL of 5M DTT to each sample and RB tube. Vortex and incubate at 56°C at 900 rpm for a minimum of 30 minutes and a maximum of O/N. Pulse spin. Resume digestion processing.	

5 Sample Selection

Generally, an individual hair or nail is received for processing and the sample is collected as described above. When multiples hairs or fingernails are to be processed as a known sample, there is a reasonable assumption of homogeneity and no sampling plan is needed.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

The quantity and quality of the DNA present within any biological material ultimately determines if a DNA extraction is successful.

A hair does that not fully digest may proceed through this procedure without additional grinding.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Procedural Specific Chemical Hazards:

- Solutions of Proteinase K can be irritating to mucous membranes. Use eye protection when handling.
- Xylene is an irritant and is toxic. Its use should be confined to a chemical fume hood whenever possible.
- Sulfuric acid is caustic. Gloves, safety glasses, and a laboratory coat must be worn whenever using sulfuric acid. Addition of sulfuric acid to the grinder must be performed inside a chemical fume hood.

10 References

FBI Laboratory Safety Manual

FBI Laboratory Quality Assurance Manual

FBI Laboratory Operations Manual

DNA Procedures Manual

Rev. #	Issue Date	History
0	02/05/16	Reformatted from Mitochondrial DNA Analysis Laboratory Procedures. Added ability to use procedure on FNC. Added option to add additional reagent and time for digesting. Increased elution volume to 65. Added option to grind if full digestion is not achieved.

Approval

 Redacted - Signatures on File

DNA Casework Unit

Procedures for Extraction of DNA with QIAamp Purification

1 Scope

These procedures describe the process for chemical digestion and purification of deoxyribonucleic acid (DNA) from body fluids and tissues (e.g., buccal swabs, blood).

2 Equipment/Materials/Reagents

Equipment/Materials

- General laboratory supplies (e.g., tubes, pipettes, centrifuge)
- Extraction basket, Costar Spin-X or equivalent
- Qiagen® QIAamp Mini Spin columns
- Qiagen® collection tubes, or equivalent
- Sonicator

Reagents

- Ethanol (EtOH), absolute
- Stain Extraction Buffer (SEB)
- Dithiothreitol (DTT), 5M solution
- Proteinase K (ProK), 20mg/mL
- Qiagen® QIAamp DNA Mini Kit
 - Qiagen® Buffer AL
 - Qiagen® Buffer AW1
 - Qiagen® Buffer AW2
- Water, Reagent Grade or equivalent
- 1% Sodium Dodecyl Sulfate (SDS), if necessary
- Xylene, if necessary
- Xylene substitute, if necessary

3 Standards and Controls

At least one extraction control (i.e., reagent blank [RB]) must be processed in parallel with each extraction batch.

For evaluation of the extraction controls, refer to the appropriate interpretation procedure of the *DNA Procedures Manual*.

4 Procedures

Refer to the DNA Procedures Introduction (DNA QA 600) for applicable general precautions and cleaning instructions.

Supplies typically needed for processing 1 sample and 1 RB (adjust for batches):

- EtOH (~1 mL), AW1 (~1.2 mL), AW2 (~1.2 mL), AL (~800 µL)
- 4 - 1.5 mL tubes
- 2 - QIAamp columns with collection tubes
- p2, p20, p200, p2000 pipettes

Tissue samples may be rinsed in any combination of the following: water, 1% SDS, xylene, xylene substitute, and/or EtOH prior to extraction based on the nature of evidence.

Record cleaning method(s) used in case notes. For additional guidance on paraffin embedded tissue, refer to the DNA collection procedure (DNA 201).

For bloodstains, measure and record the stain size in the case notes.

In general, refer to the Sampling section of this procedure and/or DNA collection procedure (DNA 201) for guidance on sample size and sample collection.

4.1	Enter appropriate barcodes. Prepare SEB + DTT by adding 7.8 µL 5M DTT to 1 mL SEB.	
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4.2	Add 300 µL of SEB+DTT and 2 µL ProK to each sample and RB tube. Vortex briefly and incubate with agitation at 56°C for 2 hours to overnight.	
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4.3 QIAamp DNA Mini Kit Purification

4.3.1	Remove tubes and pulse spin. If appropriate, transfer cutting, swab, or remaining undigested sample into a basket. Place basket into tube containing liquid extract and pulse spin. Remove and discard basket.	
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4.3.2	Add 300 µL Buffer AL. Invert mix. Incubate at 70°C for 10 minutes.	
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4.3.3	Pulse spin. Add 400 µL EtOH. Vortex mix. Pulse spin.	
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4.3.4	Add 500 µL of sample or RB to the corresponding QIAamp column within a collection tube. Spin at 8000 X g for 1 minute. Discard waste and return column to collection tube.	
4.3.5	Add remaining ~500 µL of sample or RB. Spin 8000 X g for 1 minute. Discard waste and return column to collection tube.	
4.3.6	Add 500 µL Buffer AW1. Spin 8000 X g for 1 minute. Discard waste and return column to collection tube.	
4.3.7	Add 500 µL Buffer AW2. Spin 13000-14000 X g for 3 minutes. Discard waste and return column to collection tube.	
4.3.8	Spin 13000-14000 X g for 1 minute. Transfer column to a 1.5 mL tube for elution.	
4.3.9	Add 105 µL Water. Incubate at 70°C for 5 minutes. Spin 8000 X g for 1 minute.	

Samples may be eluted in a different final volume at Examiner's discretion. If a different volume is used, record volume in case notes.

Known and tissue samples do not require mito preamplification quantitation (qPCR) and may proceed to amplification. Store extracted DNA at 4°C or colder.

Samples may be diluted in water prior to amplification. Record any dilutions in case notes. The general guide for dilution ratios are:

Buccal swab: 1:50

Blood stains: Heavy: 1:50 Light: 1:10 Gauze: 1:10

Liquid blood: 1:50

Tissue samples: 1:10

5 Sample Selection

There is a reasonable assumption of homogeneity and no sampling plan is needed. In general, the size of a sample used for extraction is as listed below. The amount of sample may vary based upon sample condition.

Buccal swab: ~ ¼ - ½ of a swab tip
Blood stain: ~3 mm x 3 mm cutting of stain
Liquid blood: ~5 µL
Tissue: ~3 mm x 3 mm x 3 mm portion

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

The quantity and quality of the DNA present within any biological material ultimately determines if a DNA extraction is successful.

9 Safety

9.1 All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material. Follow the “Safe Work Practices and Procedures,” “Bloodborne Pathogen (BBP) Exposure Control Plan (ECP),” “Personal Protective Equipment Policy,” and “Chemical Hygiene Plan” sections of the *FBI Laboratory Safety Manual*.

9.2 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures as well as the biohazardous wastes generated.

9.3 Procedural Specific Chemical Hazards:

- The sample-preparation waste contains guanidine hydrochloride from Buffers AL and AW1, which can form highly reactive compounds when combined with bleach.

- Proteinase K can be irritating to mucous membranes. Use eye protection when handling.
- Xylene is an irritant and is toxic. Its use will be confined to a chemical fume hood whenever possible.

10 References

FBI Laboratory Safety Manual

FBI Laboratory Quality Assurance Manual

FBI Laboratory Operations Manual

DNA Procedures Manual

QIAamp® DNA Mini and Blood Mini Handbook, QIAGEN®.

Rev. #	Issue Date	History
0	02/05/16	Reformatted from Mitochondrial DNA Analysis Laboratory Procedures. Added additional information for clarity. Removed instruction to add sample to tube containing reagent allowing for collection of sample prior to initiating extraction. Allowed for using the same QIAamp column collection tube. Eliminated requirement to measure final extract volume

Approval

 Redacted - Signatures on File

DNA Casework Unit

Procedures for Pre-Amplification Quantification of Mitochondrial DNA

1 Scope

These procedures apply to DNA personnel performing pre-amplification quantification of mitochondrial deoxyribonucleic acid (mtDNA) extracts. Extracts may be quantified by Real Time Quantitative PCR (qPCR) and the results may be used as a guide for subsequent amplification. The DNA Casework Unit (DCU) uses robotic workstations to automate the set-up of the quantification (aka quant) plates.

2 Equipment/Materials/Reagents

Equipment/Materials

- General laboratory supplies (e.g., tubes, pipettes, vortex, centrifuge)
- Tecan robot model 'Freedom EVO'
 - Tecan EvoWare software, version 2.3 or higher
- 7500 Sequence Detection System, Applied Biosystems
 - HID Software, version 1.2 or higher
- Microcentrifuge tubes (robot compatible)
- 96-well Plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear plate seals
- Thermal Microplate Sealer

Reagents

- mtDNA Quantitative PCR Standard Dilution Series - Double Stranded Synthetic Standard (dsT8sig), 10,000,000 copies → 10 copies, Integrated DNA Technologies
- TaqMan® Fast Advanced Master Mix, Thermo Fisher Scientific
- mtDNA Quantitative PCR Primer/Probe/IPC Mix (PPI Mix)
 - Primers (Qfor8, Qrev8, L, M, G, B), Integrated DNA Technologies
 - Probes (QRL8, C, U), Thermo Fisher Scientific
 - Internal Positive Control (IPC), Integrated DNA Technologies
- 20 pg/μL HL60 DNA (calibrator), ATCC or equivalent
- TE⁻⁴ Buffer (DNA Suspension Buffer), Fisher Scientific or equivalent
- 10% bleach, Daigger or equivalent
- 3% bleach, Molecular grade
- 70% Isopropyl alcohol, Sigma or equivalent
- Roboscrub solution, Liquinox™ or equivalent
- Purified water or equivalent, available at laboratory sinks
- Water, reagent grade or equivalent

3 Standards and Controls

The standard dilution series will be run in duplicate on each plate to generate the standard curve that is used to extrapolate the quantity of DNA in each sample. Two Master Mix (MM) controls will be run on each plate as a negative control. A TE⁻⁴ control must be processed with the first run of a prepared standard dilution series. Evaluation of these standards and controls can be found in the Data Evaluation section of this procedure.

The HL60 calibrator will be run in duplicate and is used in determining the degradation index for the samples but there is no evaluation criteria for this sample.

The reagent blank(s) (RB) associated with each sample will be quantified to determine the RB with the greatest (if any) signal.

4 Procedures

Refer to the DNA Procedures Introduction (i.e., DNA QA 600) and follow applicable general precautions and cleaning instructions.

The purified water, available via laboratory sink faucets (typically labeled DE), is used for Tecan operation and is also called Tecan system liquid.

The Mito Quant workbook is used to record the applicable case notes, to facilitate the transfer of data between instruments and equipment, and to generate examination records. Ensure the relevant run information (i.e., instruments and reagents) are recorded in the appropriate fields in the Mito Quant workbook.

4.1 Preparing the Tecan Robotic Workstation

If necessary, turn on the Tecan, which will undergo an initialization routine. Log on to the Tecan computer, launch and logon to the current Tecan software.

4.1.1	<p>Prior to daily use:</p> <ul style="list-style-type: none"> • Make ~100mL of 3% bleach to replace in front trough. • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script <p>Prior to each run:</p> <ul style="list-style-type: none"> • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. <i>When a carboy is refilled, it should be allowed to de-gas overnight before use.</i> • Check volume of waste container and empty if needed <p>As needed:</p> <ul style="list-style-type: none"> • Clean barcode scanners with a lint-free cloth
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4.2 Preparing the Sample Rack and Creating a Scan File Import

Ensure all DNA extracts and reagent blanks (aka DNA sample tubes) are in Tecan compatible tubes and appropriately barcoded. Ensure all tubes have been vortexed, spun down, and are uncapped prior to run.

4.2.1	Place DNA sample tubes in positions 1 through 16 in the sample racks. Use up to 2 sample racks (32 sample tubes), as needed. Any rack position(s) unfilled by a DNA sample tube must contain an empty tube with a unique “BL” barcode. Place sample racks in grids 4 and 5.	
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“BL” barcode tubes may be reused; however, each “BL” barcode on the Tecan must be unique.

4.2.2	Use the appropriate Tecan script to scan the sample racks and generate a .csv scan file. Import the file into the Mito Quant workbook.	
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4.3 Master Mix Preparation

This step may be performed any time prior to loading the master mix on the Tecan.

4.3.1	Prepare amplification master mix (MM) based on volumes below. Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin tubes.	
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<u>Amplification MM Component</u>	<u>μL per well*</u>
TaqMan® Fast Advanced Master Mix	10
mtDNA Quantitative PPI Mix	8

*Number of wells = [Number of sample tubes x 2] + [TE⁻⁴ control x 2, if needed]
 + [24 for standards, controls, and overage]

The PPI Mix is stored frozen until first use. Ensure the PPI Mix is labeled with the date thawed. Once thawed, the PPI Mix is stored refrigerated and may be used for up to one month.

4.4 Preparing the Tecan Deck

The steps below may be performed in any order prior to running the Tecan robot.

Positions of materials may vary between instruments. The robotic script will direct the placement.

4.4.1	Bleach/TE Rack: <ul style="list-style-type: none"> Ensure the 3% bleach solution in the front trough was replaced prior to first daily use. 	
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4.4.2	Place tubes in the Standards Rack (grid 3): <ul style="list-style-type: none"> • Positions 1 through 7: the mtDNA Quantitative PCR Standard Dilution Series • Position 8: an uncapped tube of 20 pg/μL HL60 containing at least 20 μL • Position 16: if needed, the uncapped tube of TE⁻⁴ control 	
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Before loading, vortex, quick spin, and uncapped the prepared standard dilution series and HL60, as appropriate.

The TE⁻⁴ control is provided with the mtDNA Quantitative PCR Standard Dilution Series and must be processed with the first run of the dilution series. If a TE⁻⁴ control is not needed, position 16 will be empty.

The mtDNA Quantitative PCR Standard Dilution Series may be used up to one month from the date of preparation. Prepared standards will be stored refrigerated and labeled with the preparation and expiration dates.

4.4.3	Plate Rack: <ul style="list-style-type: none"> • Place a 96-well plate into a base in the front position of the plate rack. 	
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4.4.4	Place tubes in the Master Mix Rack (grid 12): <ul style="list-style-type: none"> • Positions 3 and 4: the two tubes containing equal volumes of master mix. Ensure tubes are uncapped. • Positions 1, 2, and 5 through 16: empty tubes (with unique “BL” barcodes). 	
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4.5 Tecan Plate Preparation

4.5.1	Run the mtDNA qPCR Degradation Assay script. The Tecan will add 18 μ L of master mix and 2 μ L of each standard, sample extract, and control, in duplicate, to the 96 well plate.	
4.5.2	Seal the plate with a clear seal. Quick spin (generally ~2,000 rpm for 5 seconds).	

The seal may be applied with the Thermal Microplate Sealer or, if needed, manually. Ensure that the edges of each well are sealed.

The DNA sample tubes and standard dilution series tubes should be removed from the Tecan deck and capped prior to taking the sealed quant plate to the Amp room.

4.6 Real-Time PCR

4.6.1	Ensure the 7500 and the supporting computer are powered on. Place the sealed plate into the 7500 so that well A1 is in the back-left and the notched corner of the plate is in the back-right.	
4.6.2	In the 7500 software, open the Mito Degradation Template. (This can be done by selecting a custom assay and creating a New Experiment as From Template.) Import the file generated by the Mito Quant workbook.	
4.6.3	Save the run file, ensure the 7500 door is closed, and start the run.	

4.7 Data Evaluation

4.7.1	Using the 7500 software, review the results.	
4.7.2	Review the Standard Curve plots of C_T (cycle threshold) versus Quantity (DNA concentration). Use the Target dropdown menu to view the “QRL” curve results. Record the results for the slope, R^2 , and Y-intercept in the Mito Quant workbook.	
4.7.2.1	A passing run will have: <ul style="list-style-type: none"> • $R^2 \geq 0.985$ • Slope in the range of -3.200 and -3.600 • Y-intercept in the range of 36.100 and 39.600 	
4.7.2.2	If the R^2 value is < 0.985 , if the slope is out of range, or if there is a visible outlier, omitting a poor replicate of a standard(s) and reanalyzing may result in passing values for the standard curve. (<i>For each standard pair in the dilution series, only one of the replicates may be omitted if necessary</i>).	

To omit a replicate:

Right click on the well and choose omit, then reanalyze the data. (This may be done by clicking the green “Analyze” tab in the top right corner.)

4.7.2.3	If the R^2 , slope, or Y-intercept do not meet the required values, the plate fails, the data is not suitable for evaluation, and the samples must be requanted.	
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If two or more wells are omitted from the standard curve or if the R^2 , slope, or Y-intercept do not meet the required values, the standard dilution series should be discarded.

4.7.3	Export the results from the 7500. (<i>This may be done by selecting Export from File menu, then choosing Results.</i>) Import the results file (.txt) into the Mito Quant workbook.	
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4.7.4	Check the quantification results and internal PCR control (IPC) C _T of the Master Mix control. <ul style="list-style-type: none"> • The Master Mix control should display no quantifiable DNA. • If a DNA value of greater than 10 copies/μL appears in the master mix control, a contaminant may be present. 	
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4.7.5	For plates using a new DNA standard dilution series, check the quantification result and IPC C _T of the TE ⁻⁴ Control. <ul style="list-style-type: none"> • If a DNA value of greater than 10 copies/μL appears in the TE control, a contaminant may be present. 	
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If a DNA value of greater than 10 copies/μL appears in any of the negative controls, the samples may be re-quanted at examiner discretion.

If the HL60 calibrator does not yield results, the samples may be re-quanted at examiner discretion.

4.7.6	The sample data should be evaluated for: <ul style="list-style-type: none"> • an indication of possible inhibition based on the IPC. • an indication of degradation based on the degradation index (ΔΔCT). An examiner will review the quantification results and determine the mtDNA region(s) to amplify and the primers to use for amplification. 	
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The IPC is generally expected to be between 29 and 31. An IPC >31 may indicate inhibition.

Samples that have an indication of possible inhibition may be diluted and re-quanted. Reagent grade water is used to dilute samples as appropriate. Any dilution(s) made will be recorded in the case notes.

5 Sampling

Not applicable.

6 Calculations

$$\Delta C_T = C_{T\ 300} - C_{T\ 100}$$

$$\Delta\Delta C_T = \Delta C_{T\ \text{sample}} - \Delta C_{T\ \text{HL60 calibrator}}$$

7 Measurement Uncertainty

Not applicable.

8 Limitations

The accuracy of the results obtained from qPCR are dependent upon the precision of the standard curve and the results should be reviewed in accordance with the parameters previously listed in the Data Evaluation section of this document. The resulting values are an estimate of the quantity of DNA in the sample.

9 Safety

Refer to the *FBI Laboratory Safety Manual*.

10 References

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. *Applied Biosystems 7300/7500/7500 Fast Real-Time PCR System Absolute Quantitation Using Standard Curve Getting Started Guide*. 2006.

Applied Biosystems. *Applied Biosystems 7500/7500 Fast Real-Time PCR System Maintenance Guide*. 2010.

Kavlick, M.F. *Development of a triplex mtDNA qPCR assay to assess quantification, degradation, inhibition, and amplification target copy numbers*. Mitochondrion, *in press*. Available online at: <https://doi.org/10.1016/j.mito.2018.09.007>

Rev. #	Issue Date	History
0	02/05/16	Reformatted from Mitochondrial DNA Analysis Laboratory Procedures. qPCR Master Mix is now prepared in advance and instructions are in the QA reagent SOP and up to 4 freeze/thaws are approved. Extended allowable usage of previously prepared standards to up to 9 freeze/thaws.
1	02/15/19	Overhauled entire procedure for the new degradation assay including added sections to incorporate automated procedures. Added quality control procedures to Appendix A.

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 02/14/2019

DCU Chief

Date: 02/14/2019

QA Approval

Quality Manager

Date: 02/14/2019

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency of performance verification and additional requirements.

A. General Maintenance of the AB 7500 Sequence Detection System

For semi-annual general maintenance, refer to the instructions in the Applied Biosystems *7500/7500 Fast Real-Time PCR System Maintenance Guide* to perform the following:

1. Regions of Interest (ROI) Calibration (Chapter 2)
2. Background Calibration and Optical Calibration (Chapter 3)
3. Dye Calibrations (Chapter 4) for standard dyes VIC, FAM, and NED

B. Performance Verification of the AB 7500 Sequence Detection System

The performance verification of the AB 7500 Sequence Detection System can be accomplished by running either the Quantifiler® DUO DNA Quantification Kit or the mtDNA qPCR Degradation Assay as both assays use the same dyes (FAM, VIC, NED). Instructions for performance verification using Quantifiler® DUO are in the procedure for quantitation of nuclear DNA (i.e., DNA 226). Follow the below instructions for running the performance verification with the mtDNA qPCR Degradation Assay:

1. Using in-use lots of reagents for the mtDNA qPCR Degradation Assay, run a plate containing the mtDNA Quantitative PCR Standard Dilution Series, the HL60 calibrator, and appropriate controls, all in duplicate.
2. The 7500 will be deemed suitable for casework analysis if:
 - a. The slope is within the acceptable range of -3.200 to -3.600.
 - b. The R^2 is ≥ 0.985 .
 - c. The Y-intercept is within the acceptable range of 36.100 to 39.600

C. General Maintenance of the Tecan Robotic Workstation

RoboScrub cleaning should be performed weekly, generally at the end of a workday:

1. Make ~3.5 L of diluted Liquinox (see instructions on the label of the bottle for preparation)
2. ~3.5 L purified water in a separate container is needed
3. Run the RoboScrub Clean script, and follow the prompts

D. Performance Verification of the Tecan Robotic Workstation

1. An Artel MVS Multichannel Verification System and NIST traceable standards will be used to test the accuracy and precision of the liquid handling by the Tecan. Refer to the *Artel MVS Multichannel Verification System User Guide* for operation of the Artel MVS.
2. The Tecan Robotic workstations are typically configured with eight (8) fixed tips and there are multiple volumes aliquoted during each procedure. A minimum of 6 repetitions will be performed with each tip for each volume.
3. The results must be within the tolerance limits set by DCU for each volume. At times, it may be necessary to modify/optimize the Tecan liquid class parameters (e.g., offset and factor).
4. If the performance verification of the Tecan does not meet the above listed criteria, the performance verification will be repeated. If the results are still deemed unsuitable, then the Technical Leader will be consulted.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

A. Performance Verification of the mtDNA qPCR Degradation Assay Reagents

Each new lot of dsT8sig standard secondary stock, TaqMan® Fast Advanced Master Mix, and mtDNA Quantitative PCR Primer/Probe/IPC Mix (PPI Mix) will be evaluated by running the standard dilution series, the HL60 calibrator, and appropriate controls, all in duplicate. The new lot of reagents will be deemed suitable for use in casework if:

- a. The slope is within the acceptable range of -3.200 to -3.600.
- b. The R^2 is ≥ 0.985 .
- c. The Y-intercept is within the acceptable range of 36.100 to 39.600

More than one of the above reagents may be simultaneously tested for reliability.

Procedures for the Amplification and Amplicon Purification of Mitochondrial DNA

1 Scope

These procedures apply to DNA personnel who perform amplification of mitochondrial deoxyribonucleic acid (mtDNA) via the polymerase chain reaction (PCR) and purification of amplicons and to DNA personnel that perform the associated quality control procedures.

2 Equipment/Materials/Reagents

Equipment/Materials

- Thermal cycler, ABI 9700 or equivalent
- General laboratory supplies (e.g. tubes, pipettes, vortexer)
- PCR tube rack
- Tecan robot model 'Freedom EVO'
 - Tecan EvoWare software, version 2.3 or higher
- 96-well plates, Applied Biosystems MicroAmp® optical or equivalent
- Clear and/or foil plate seals
- Thermal microplate sealer

Reagents

- Water, Reagent Grade or equivalent
- GeneAmp® 10X PCR Buffer I
- Bovine Serum Albumin (BSA), 1.6 µg/µL
- GeneAmp® Deoxyribonucleotide Triphosphates (dNTP) blend, 2.5 mM each
- mtDNA Amplification Primers, 30 µM or 10 µM
- AmpliTaq Gold®, 5U/µl
- Positive control DNA, HL60 or equivalent
- ExoSAP-IT®
- 3% bleach (household or equivalent)
- 10% bleach (Daigger or equivalent)
- Isopropyl alcohol, 70%
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution (Liquinox™ or equivalent)

3 Standards and Controls

At least one negative control (NC) and one positive control (i.e., HL60) must be processed concurrently in the same instrument with the samples for each amplified region. An associated reagent blank (RB) must be amplified with the same primers and at the most sensitive concentration conditions as required by the sample(s) containing the least amount of DNA.

For evaluation of the amplification controls, refer to the appropriate interpretation procedure of the *DNA Procedures Manual*.

4 Procedures

Refer to DNA QA 600 in the *DNA Procedures Manual* for applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water will be used. The purified water available via faucets (typically labeled DE) at the laboratory sinks, is used for Tecan operation and is also called Tecan system liquid.

The following information, along with the pre-amplification quantitation (qPCR) results and/or autosomal DNA results should be used to determine the mtDNA region to amplify and the primers to use. The two primers listed represent the light (L) and heavy (H) strand primers that are added to the master mix for the amplification of the listed region.

1. For samples from which abundant amounts of mtDNA template are expected, the whole control region (WCR) can be amplified using primer sets listed below. This amplification may be done using the Tecan robot platform.

<u>Region</u>	<u>Primers</u>
WCR	A1, 617

2. To obtain the maximum possible sequence from a sample, flexibility in the choice of primer sets is allowed. This may include the two hypervariable (HV) regions, HV1 and HV2, listed below.

<u>Region</u>	<u>Primers</u>
HV1	A1, B1
HV2	C1, D1

3. For samples from which lower amounts of mtDNA template are expected, HV1A, HV1B, HV2A, and HV2B can be amplified using primer sets listed below.

<u>Region</u>	<u>Primers</u>
HV1A	A1, B2
HV1B	A2, B1
HV2A	C1, D2
HV2B	C2, D1

4. For samples from which extremely degraded DNA template is expected, MPS1-MPS5 can be amplified using mini-primer sets (MPS) listed below.

<u>Region</u>	<u>Primers</u>
MPS1	1A, 1B
MPS2	2A, 2B
MPS3	3A, 3B
MPS4	4A, 4B
MPS5	5A, 5B

5. For samples that have been extracted on the EZ1, only WCR or HV1/HV2 can be used. For samples that have been extracted on the QIASymphony, only WCR can be used.

4.1 Amplification with WCR

4.1.1	Prepare WCR amplification master mix.	
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Typically, a 5-reaction master mix is sufficient to account for a sample, RB, NC, positive control, and appropriate overage. When amplifying a batch of samples, the volume of master mix should be calculated to account for the number of samples, RB(s), NC, positive control, and overage. Components should be added in order listed below.

Master Mix Component	µL per Sample	µL for 5 reactions
Water	12.0	60.0
10X PCR Buffer	5.0	25.0
BSA (1.6 µg/µL)	5.0	25.0
dNTP mix	4.0	20.0
A1 primer 30 µM	1.0	5.0
617 primer 30 µM	1.0	5.0
AmpliTag Gold	2.0	10.0
TOTALS	30.0	150

4.1.2	Add 30 µL of master mix to each tube.	
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4.1.3	Add 20 µL of sample extract (or extract dilution) to the sample tube. Add 20 µL of RB (or RB dilution) to the RB tube. Add 20 µL of positive control DNA to the positive control tube. Add 20 µL of water to the NC tube.	
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When amplifying a batch of samples, add all samples and applicable blanks prior to adding the positive and negative controls.

4.1.4	<p>Place tubes in the thermal cycler. Start the “WCR32” or “WCR36” program:</p> <ul style="list-style-type: none"> • 95°C for 9 minutes • 32 or 36 cycles: <ul style="list-style-type: none"> ○ 95°C for 10 seconds ○ 53°C for 30 seconds ○ 72°C for 30 seconds • 72°C for 10 minutes • 4°C hold indefinitely 	
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Record of the thermal cycler program used will be in the case notes.

Amplicons can then be purified using ExoSAP-IT.

4.2 Automated Amplification with WCR

4.2.1	<p>Ensure the Tecan is prepared to run.</p> <p>Prior to daily use:</p> <ul style="list-style-type: none"> • Make ~100 mL of 3% bleach to replace bleach in <i>front</i> trough • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script <p>Prior to each run:</p> <ul style="list-style-type: none"> • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. <i>When a carboy is refilled, it should be allowed to de-gas overnight before use.</i> • Check volume of waste container and empty if needed <p>Weekly, generally at the end of the workday:</p> <ul style="list-style-type: none"> • Make ~3.5 L of diluted Liquinox solution (see instructions on bottle) • ~3.5 L purified water in a separate container is needed • Run the RoboScrub Clean script, and follow the prompt 	
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The daily start up script prompt “Check syringes and tips,” refers to checking that the tubing and syringes (plunger lock screws) are tight and not introducing air bubbles, and that the tips are tight, free of clogs, and not leaking.

4.2.2	<p>Create the appropriate volume of master mix based on the number of samples to be amplified (with overage). Equally distribute the master mix between two labeled microcentrifuge tubes. Vortex and quick spin.</p>	
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This step may be performed any time prior to loading the master mix on the Tecan robot.

When amplifying a batch of samples, the volume of master mix should be calculated to account for the number of samples, RB(s), NC, positive control, and an overage of two samples per amplification plate. Components should be added in order listed below.

Master Mix Component	μL per Sample
Water	12
10X PCR Buffer	5
BSA (1.6 μg/μL)	5
dNTP mix	4
A1 primer (30 μM)	1
617 primer (30 μM)	1
AmpliTaq Gold	2
TOTALS	30

4.2.3	<p>Prepare the Tecan Deck. <i>The steps below may be performed in any order prior to running the Tecan robot.</i></p> <p>Bleach Rack:</p> <ul style="list-style-type: none"> • Ensure the 3% bleach solution in the front trough was replaced prior to daily use. <p>Plate Rack:</p> <ul style="list-style-type: none"> • Place a 96-well plate into a base. Place into the rear position of the plate rack. <p>Master Mix Rack:</p> <ul style="list-style-type: none"> • Place two uncapped tubes containing equal volumes of master mix in positions 3 and 4. Place empty tubes in positions 1-2 and 5-16. <p>Sample Rack:</p> <ul style="list-style-type: none"> • Ensure all DNA extracts, reagent blanks, and positive controls are in Tecan compatible tubes. • Place uncapped DNA sample tubes in the desired sequence starting in position 1 of sample rack 1 and continue on to additional racks as needed. 	
4.2.4	<ul style="list-style-type: none"> • Run Scan Script and import scan file into workbook. 	
4.2.5	<p>Run Amplification Script.</p> <ul style="list-style-type: none"> • Follow the prompts to ensure the appropriate racks are on the deck as required. • Enter number of samples to be amplified when prompted. • Each sample tube should contain at least 22 μL, including positive and negative controls. 	

The Tecan will prepare the amp plate so that each well contains 30 μL of master mix and 20 μL of DNA template or control.

4.2.6	Upon completion of the Tecan run, seal the amplification plate with a clear or foil seal.	
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The seal may be applied with the Thermal Microplate Sealer or, if necessary, manually. Ensure that the edges of each well are properly sealed.

4.2.7	Quick spin (generally ~2,000 rpm for 5 seconds).	
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Sample tubes should be removed from the Tecan deck and capped prior to proceeding to the Amplification (Amp) room.

4.2.8	Proceed to amp room. Place the amp plate in an appropriate thermal cycler. Place an optical compression pad, gold side up, onto the top of the sealed plate and close the lid by pressing the lever down completely.	
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4.2.9	Start the “WCR32” or “WCR36” program: <ul style="list-style-type: none"> • 95°C for 9 minutes • 32 or 36 cycles: <ul style="list-style-type: none"> ○ 95°C for 10 seconds ○ 53°C for 30 seconds ○ 72°C for 30 seconds • 72°C for 10 minutes • 4°C hold indefinitely 	
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Record of the thermal cycler program used will be in the case notes.

4.3 Amplification of HV1, HV2, HV1A, HV1B, HV2A, HV2B

4.3.1	Prepare an amplification master mix for each region.	
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Typically, a 5-reaction master mix is sufficient to account for sample, RB, NC, positive control, and appropriate overage. Components should be added in order listed below.

Master Mix Component	μL per Sample	μL for 5 reactions
Water	6.0	30.0
10X PCR Buffer	2.5	12.5
BSA (1.6 μg/μL)	2.5	12.5
dNTP mix	2.0	10.0
(L) primer 30 μM	0.5	2.5
(H) primer 30 μM	0.5	2.5
AmpliTaq Gold	1.0	5.0
TOTALS	15.0	75.0

4.3.2	Add 15 μL of the appropriate master mix to the respective tubes.	
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4.3.3	Add 10 μL of sample extract (or sample dilution) to each sample tube. Add 10 μL of RB (or RB dilution) to each RB tube. Add 10 μL of positive control DNA to each positive control tube. Add 10 μL of water to each NC tube.	
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To use additional extract volume, see the section for using 16 μL of extract.

4.3.4	Place tubes in the thermal cycler. Start the "TAQ 32" or "TAQ 36" program: <ul style="list-style-type: none"> • 95°C for 9 minutes • 32 or 36 cycles: <ul style="list-style-type: none"> ○ 95°C for 10 seconds ○ 60°C for 30 seconds ○ 72°C for 30 seconds • 4°C hold indefinitely 	
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Record of the thermal cycler program used will be in the case notes.

Amplicons can then be purified using ExoSAP-IT.

4.4 Amplification with Mini-primer Sets

4.4.1	Prepare an amplification master mix for each mini-primer set.	
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Typically, a 5-reaction master mix is sufficient to account for the sample, RB, NC, positive control, and appropriate overage. Components should be added in order listed below.

Master Mix Component	μL per Sample	μL for 5 reactions
Water	6.0	30.0
10X PCR Buffer	2.5	12.5
BSA (1.6 $\mu\text{g}/\mu\text{L}$)	2.5	12.5
dNTP mix	2.0	10.0
(L) primer 10 μM	0.5	2.5
(H) primer 10 μM	0.5	2.5
AmpliTag Gold	1.0	5.0
TOTALS	15.0	75.0

4.4.2	Add 15 μL of the appropriate master mix to respective tubes.	
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4.4.3	Add 10 μL of sample extract, or extract dilution, to each sample tube. Add 10 μL of RB, or RB dilution, to each RB tube. Add 10 μL of positive control DNA to each positive control tube. Add 10 μL of water to each NC tube.	
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To use additional extract volume, see the section for using 16 μL of extract.

4.4.4	Place tubes in the thermal cycler. Start "MINI" program: <ul style="list-style-type: none"> 95°C for 12 minutes 36 cycles: <ul style="list-style-type: none"> 95°C for 15 seconds 56°C for 30 seconds 72°C for 45 seconds 4°C hold indefinitely 	
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Amplicons can then be purified using ExoSAP-IT.

4.5 Amplification Using 16 μ L of Extract

Increased sample volume is appropriate for amplifications of HV1A, HV1B, HV2A, HV2B, HV1, HV2 or the Mini-primer sets.

4.5.1	Prepare an amplification master mix for each primer set.	
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Typically, a 5-reaction master mix is sufficient to account for the sample, RB, NC, positive control, and appropriate overage. Components should be added in order listed below.

Master Mix Component	μ L per Sample	μ L for 5 reactions
10X PCR Buffer	2.5	12.5
BSA (1.6 μ g/ μ L)	2.5	12.5
dNTP mix	2.0	10.0
(L) primer 10 μ M	0.5	2.5
(H) primer 10 μ M	0.5	2.5
AmpliTag Gold	1.0	5.0
TOTALS	9.0	45.0

4.5.2	Add 9 μ L of the appropriate master mix into respective tubes.	
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4.5.3	Add 16 μ L of sample extract to each sample tube. Add 16 μ L of RB to each RB tube. Add 16 μ L of positive control DNA to each positive control tube. Add 16 μ L of water to each NC tube.	
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If needed, water may be added to a sample and/or RB in order to obtain a total volume of 16 μ L. (For example: If only 15 μ L of sample extract is available, 1 μ L water may be added to the sample tube.) However, water cannot be added to the RB only.

4.5.4	Place tubes in the thermal cycler. Start appropriate program: For HV1A, HV1B, HV2A, HV2B, HV1, HV2, use "TAQ36" For Mini-primers, use "MINI"	
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Record of the thermal cycler program used will be in the case notes.

Amplicons can then be purified using ExoSAP-IT.

4.6 Amplicon Purification by ExoSAP-IT

4.6.1	<p>Add ExoSAP-IT to the amplicon tubes: For standard or mini-primer sets, add 5 µL For WCR sets, add 10 µL Vortex or pipette mix. Place tubes in the thermal cycler and run “EXOSAPIT” program:</p> <ul style="list-style-type: none"> • 37°C for 15 minutes • 80°C for 15 minutes • 4°C hold indefinitely 	
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Samples are ready for post-amplification quantitation.

If needed based on the sequencing results, this step may be repeated and the sample(s) re-processed as appropriate.

4.7 Automated Amplicon Purification by ExoSAP-IT

4.7.1	<p>Ensure the Tecan is prepared to run.</p> <p>Prior to daily use:</p> <ul style="list-style-type: none"> • Make ~100 mL of 3% bleach to replace bleach in <i>center</i> trough • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script <p>Prior to each run:</p> <ul style="list-style-type: none"> • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. <i>When a carboy is refilled, it should be allowed to de-gas overnight before use.</i> • Check volume of waste container and empty if needed <p>Weekly, generally at the end of the workday:</p> <ul style="list-style-type: none"> • Make ~3.5 L of diluted Liquinox solution (see instructions on bottle) • ~3.5 L purified water in a separate container is needed • Run the RoboScrub Clean script, and follow the prompt 	
4.7.2	<p>Set up Tecan deck:</p> <ul style="list-style-type: none"> • Bleach Rack: Ensure the 3% bleach solution in the <i>center</i> trough was replaced prior to daily use. • Plate Rack: Spin down 96-well amplification plate and carefully remove seal. Place into the rear position of the plate rack. • ExoSAP-IT Rack: Vortex and spin down one tube of ExoSAP-IT. Place into position 1 of tube rack. 	

4.7.3	Start the ExoSAP-IT script. The script will prompt the user to enter the number of occupied wells in the amplification plate. The Tecan will add 10 μ L of ExoSAP-IT to each well indicated and mix thoroughly.	
4.7.4	Seal plate. Place an optical compression pad, gold side up, onto the top of the sealed plate and close the lid by pressing the lever down completely. Place in the thermal cycler, and run “EXOSAPIT” program: <ul style="list-style-type: none"> • 37°C for 15 minutes • 80°C for 15 minutes • 4°C hold indefinitely 	

Samples are ready for cycle sequencing. *Post-amplification quantitation is not required for samples processed with the automated procedures.*

If needed based on the sequencing results, this section may be repeated and the sample(s) re-processed as appropriate.

5 Sampling or Sample Selection

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 The post-amplification yields of the positive and negative amplification controls indicate whether the amplification process is successful.

8.2 Samples that have been extracted on the EZ1 are only approved for amplification of WCR and HV1/HV2. Samples that have been extracted on the QIASymphony are only approved for amplification of WCR.

9 Safety

9.1 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures.

10 References

FBI Laboratory Quality Assurance Manual

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. GeneAmp® PCR System 9700 User's Manual Set. 1997.

Rev. #	Issue Date	History
0	02/05/16	Reformatted from Mitochondrial DNA Analysis Laboratory Procedures. Referenced DNA QA 600 for general guidelines and cleaning. Changed order of sample addition to sample, RB, Pos Amp, Neg Amp. Deleted sample storage info contained in DNA QA 601. Consolidated WCR for single samples and batching. Added limitations for samples extracted with EZ1 and QIASymphony.
1	02/28/18	Adjusted scope to apply to DNA personnel Adjusted applicability of primer set section. Added guidance about appropriate water to section 4. Added automated procedures as sections 4.2 and 4.7. Renumbered existing sections. Added allowance for repeating ExoSAP-IT process Added Appendix A from Mito QC instructions in STACS

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 02/27/2018

DCU Chief

Date: 02/27/2018

DSU Chief

Date: 02/27/2018

QA Approval

Quality Manager

Date: 02/27/2018

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency of performance verifications and additional requirements.

A. Performance Verification (PV) of the Tecan Robotic Workstation

Refer to the nuclear DNA quantification procedure (i.e., nDNA 226) for instructions for the PV of the Tecan Robotic Workstation.

B. Performance Verification of the 9700 Thermal Cycler

1. Cleaning - Refer to Cleaning the sample wells and Cleaning the sample block cover procedures described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set.
2. Temperature Verification Test - This procedure verifies that the thermal cycler remains within the temperature accuracy specification. Refer to Running the Temperature Verification Test procedure described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set. This test procedure requires the use of a Temperature Verification System.
3. Temperature Non-uniformity Test - This procedure verifies the temperature uniformity of the sample wells in the thermal cycler. Refer to Running the Temperature Non-uniformity Test procedure described in the Maintenance section of the GeneAmp® PCR System 9700 User's Manual Set. This test procedure requires the use of a Temperature Verification System.
4. Rate Test and Cycle Test - These procedures verify the integrity of the cooling and heating system of a thermal cycler. Refer to Running System Performance Diagnostics procedure described in the 96-Well Sample Block Module section of the GeneAmp® PCR System 9700 User's Manual Set.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

A. Amplification Reagents

The following reagents will be tested by amplifying a less sensitive region (generally HV2b) of a positive control sample including a NC, quantification by capillary electrophoresis and sequencing. Reagents may be concurrently tested or tested with an in use lot of the counterpart

reagent(s). Multiple positive control samples may be amplified; at least one must yield the expected positive control sequence.

1. 10X PCR Buffer
2. AmpliTaq Gold
3. Deoxyribonucleotide triphosphate mix (dNTPs)
4. Bovine Serum Albumin (BSA)
5. EXOSap-IT

B. Positive Control

New lots and dilutions of HL60 DNA will be tested by amplification of the WCR, including a NC, quantification by capillary electrophoresis, and sequencing. The HL60 DNA will be diluted to a final concentration of 20 pg/μL.

C. Amplification Primers

New lots of amplification primers will be tested by amplifying the HL60 DNA with all of the appropriate primer pairs used with the primer. The resulting amplicon(s) will be quantified by capillary electrophoresis and sequenced with all primers typically used in the amplicon(s).

Primer for QC	Amplification Regions
A1	HV1, HV1a, WCR
A2	HV1b
B1	HV1, HV1b
B2	HV1a
C1	HV2, HV2a
C2	HV2b
D1	HV2, HV2b
D2	HV2a
617	WCR

DNA Casework Unit

Procedures for Post-Amplification Quantification of Mitochondrial DNA

1 Scope

These procedures describe the process for quantification of amplified mitochondrial deoxyribonucleic acid (mtDNA). Following post-amplification purification, amplicons are quantified with Agilent 2100 Bioanalyzer and DNA1000 or 7500 Series II LabChip kit.

2 Equipment/Materials/Reagents

Equipment/Materials

- General laboratory supplies (e.g., pipettes, centrifuge)
- 2100 Bioanalyzer, Agilent Technologies
 - 2100 Expert software (Rev. B.02.03 or above).
- Bioanalyzer Chip priming station, Agilent Technologies
- Vortex mixer
- DNA LabChip®, Agilent Technologies

Reagents

- DNA1000 or 7500 Kit, Agilent Technologies.
 - DNA1000 or 7500 Dye concentrate, if needed
 - DNA1000 or 7500 DNA gel matrix, if needed
 - DNA1000 or 7500 gel-dye mix
 - DNA1000 or 7500 markers
 - DNA1000 or 7500 ladder
- Water, Reagent Grade or equivalent

3 Standards and Controls

All corresponding reagent blanks (RB) and amplification controls (Negative Control [NC] and Positive control [i.e., HL60]) to a sample must be quantified.

For evaluation of the quantification data, refer to the last section of this procedure.

4 Procedures

Refer to DNA Procedures Introduction (DNA QA 600) and follow applicable general precautions and cleaning instructions.

4.1 DNA 1000 Series II Kit

The DNA 1000 kit is typically used for samples amplified with HV1A, HV1B, HV2A, HV2B, HV1, HV2 or mini-primer sets.

4.1.1 DNA 1000 Gel-Dye Mix Preparation

4.1.1.1	Allow dye concentrate and DNA gel matrix to equilibrate to room temperature for approximately 30 minutes. Add 25 µL of dye concentrate into DNA 1000 gel matrix vial. Cap and vortex well.	
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4.1.1.2	Transfer gel-dye mix to spin filter. Spin at approximately 2240 X g for 15 minutes. Discard spin filter. Label tube (include date of preparation).	
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Use gel-dye mix within 4 weeks of preparation. Protect from light and store at 4°C.

4.1.2 DNA 1000 Chip Loading

4.1.2.1	Add 9 µL gel-dye mix to the well marked “ G ” (Circled G). Place chip into priming station (syringe clip adjusted to lowest notch) with syringe set at 1 mL. Close priming station and press plunger until held by syringe clip. Wait 60 seconds, then release clip and pull back syringe plunger to 1 mL. Open priming station and remove chip.	
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4.1.2.2	Add 9 µL gel-dye mix to 2 wells marked “G”. Add 1 µL of DNA 1000 ladder (yellow tube) to ladder well. Add 5 µL of DNA 1000 markers (green tube) to ladder well. Add 5 µL of DNA 1000 markers (green tube) to each sample well to be used. Add 6 µL of DNA 1000 markers (green tube) to each unused sample well. Add 1 µL control/sample to appropriate wells.	
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4.1.2.3	Place chip into vortex mixer and vortex for 60 seconds at 2400 rpm (speed may be lowered).	
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The chip should be run within 5 minutes of preparation to avoid failure due to evaporation.

4.2 DNA 7500 Series II Lab Kit

The DNA 7500 kit is typically used for samples amplified with WCR primers.

4.2.1 DNA 7500 Gel-Dye Mix Preparation

4.2.1.1	Allow dye concentrate and DNA gel matrix to equilibrate to room temperature for approximately 30 minutes. Add 25 µL of dye concentrate into DNA 7500 gel matrix vial. Cap and vortex well.	
4.2.1.2	Transfer gel-dye mix to spin filter. Spin at approximately 1500 X g for 10 minutes. Discard spin filter. Label tube (include date of preparation).	

Use gel-dye mix within 4 weeks of preparation. Protect from light and store at 4°C.

4.2.2 DNA 7500 Chip Loading

4.2.2.1	Add 9 µL gel-dye mix to well marked “ G ”. (Circled G) Place chip into priming station (syringe clip adjusted to highest notch) with syringe set at 1 mL. Close priming station and press plunger until held by syringe clip. Wait 30 seconds, then release clip and pull back syringe plunger to 1 mL. Open priming station and remove chip.	
4.2.2.2	Add 9 µL gel-dye mix to 2 wells marked “G”. Add 1 µL of DNA 7500 ladder (yellow tube) to ladder well. Add 5 µL of DNA 7500 markers (green tube) to ladder well. Add 5 µL of DNA 7500 markers (green tube) to each sample well to be used. Add 6 µL of DNA 7500 markers (green tube) to each unused sample well. Add 1 µL control/sample to appropriate wells.	
4.2.2.3	Place chip into vortex mixer and vortex for 60 seconds at 2400 rpm (speed may be lowered).	

The chip should be run within 5 minutes of preparation to avoid failure due to evaporation.

4.3 Quantification Using Agilent 2100 Bioanalyzer

4.3.1	Turn on Bioanalyzer and computer. Start software by double-clicking 2100 Expert icon. Click on Instrument context and select Bioanalyzer. Insert chip into Bioanalyzer and close lid.	
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Screen should now display a chip in upper left. If a chip is not displayed, remove chip from instrument and inspect wells for appropriate volume.

4.3.3	Select DNA 1000 II or DNA 7500 II from Assay Selection button at upper right of Instrument screen. Enter Laboratory number (and sample name, if possible) in file prefix box in Destination section. Enter number of wells to be run. Click Start button at upper right (under Assay Selection button). Fill out Sample Information table by clicking on “Data File: name” or by clicking on Data and Assay icon in Contexts panel.	
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4.3.4	When run is complete, place electrode cleaner (containing 350 µL water) in Bioanalyzer and close lid for 10 seconds. Open the lid, remove electrode cleaner, and let dry 10 seconds. Close lid.	
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4.4 Evaluation of Results

4.4.1 The software calculates sample concentration. Multiple peak concentrations should be added together when appropriate. If a single peak concentration exceeds 50 ng/µL, a dilution will be prepared and quantified.

4.4.2 If a peak of interest is not integrated, it can be manually integrated by decreasing analysis thresholds. In most instances decreasing Height Threshold is sufficient for integration. If this is not successful, decrease Height Threshold and adjust one or more thresholds as detailed below.

Click on expansion tab on right side of sample electropherogram panel and select LOCAL tab. Change value of following threshold(s) as appropriate and press ENTER

- Height Threshold (default value = 20) can be decreased to between 19 – 0.02
 - Slope Threshold (default value = 0.5) can be decreased to between 0.45 – 0.05
 - Area Threshold (default value = 0.1) can be decreased to between 0.09 – 0.02
 - Width Threshold (default value = 0.5) can be decreased to between 0.45 – 0.1
- Save file and reprint sample of interest if necessary.

Do NOT manually integrate the peak of interest by changing the start and stop points for integration on the electropherogram of the peak of interest.

4.4.3 Samples may proceed to cycle sequencing of a region when:

- The sample and positive control concentrations are ≥ 1 ng/ μ L.
AND
- The NC and RB concentrations are $< 10\%$ of the corresponding sample concentration.

Samples that do not meet these criteria will not be taken on to cycle-sequencing. Sample and positive control concentrations may be rounded up from 0.96 ng/ μ L.

5 Sampling

Not applicable.

6 Calculations

Not applicable.

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 If a single peak concentration exceeds 50 ng/ μ L, a dilution will be prepared and quantified.

8.2 Minimum sample and positive control concentration and maximum RB and NC concentrations are empirically determined to provide a level at which the cycle-sequence data is expected to not be interpretable.

9 Safety

9.1 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures.

9.2 Procedural Specific Chemical Hazards:

- Agilent kit components contain dimethyl sulfoxide (DMSO). This dye binds to nucleic acids and is treated as a potential mutagen.

10 References

FBI Laboratory Safety Manual

DNA Procedures Manual

Agilent 2100 Bioanalyzer 2100 Expert User's Guide. Available online at www.agilent.com.

Agilent 2100 Bioanalyzer Maintenance and Troubleshooting Guide. Available online at www.agilent.com

Rev. #	Issue Date	History
0	02/05/16	Reformatted from Mitochondrial DNA Analysis Laboratory Procedures.

Approval


Redacted - Signatures on File

DNA Procedures for Cycle-Sequencing, Centri-Sep Purification, and Sequencing of mtDNA

1 Scope

These procedures apply to DNA personnel who perform cycle-sequencing, Centri-Sep purification and sequencing of amplified mitochondrial deoxyribonucleic acid (mtDNA).

2 Equipment/Materials/Reagents

Equipment/Materials

- Thermal cycler, ABI 9700
- Centri-Sep 8 Strip Spin Columns, Princeton Separations or equivalent
- Disposal plate
- 3130xl Genetic Analyzer, Life Technologies
 - Data Collection, version 4.0 or greater, Life Technologies
- General laboratory supplies (e.g., pipettes, tubes)
- Tecan robot model 'Freedom EVO'
 - Tecan EvoWare software, version 2.3 or higher
- 96-well plates, Applied Biosystems MicroAmp® optical or equivalent
- Plate septa, Applied Biosystems or equivalent
- Clear and/or foil plate seals
- Thermal microplate sealer

Reagents

- BigDye® Terminator v1.1 Cycle Sequencing Kit, Applied Biosystems
- Primers, 1 µM
- 1X Genetic Analyzer Buffer
- Polymer, POP-6™
- Water, reagent grade or equivalent
- 3% bleach (household or equivalent)
- 10% bleach (Daigger or equivalent)
- Isopropyl alcohol, 70%
- Purified water or equivalent, available at laboratory sinks
- RoboScrub solution (Liquinox™ or equivalent)
- Bigdye® v1.1 Sequencing Standard, Applied Biosystems
- Hi-Di Formamide

3 Standards and Controls

At least one corresponding reagent blank (RB), positive amplification control (i.e., HL60) and negative amplification control (NC) must be processed in parallel for the cycle-sequencing of each sample. These corresponding reagent blanks and amplification controls of a sample must be sequenced.

Re-cycle sequencing must include appropriate positive and negative controls in addition to any sample and/or RB which is determined by the Examiner to need re-cycle sequencing (See section 4.9 for more information on re-cycle sequencing).

For evaluation of the sequencing data, refer to the appropriate interpretation procedure (i.e., DNA 410) of the *DNA Procedures Manual*.

4 Procedures

Refer to the DNA Procedures Introduction (i.e., DNA QA 600) and follow applicable general precautions and cleaning instructions.

For water that will come into contact with the DNA samples (e.g., for dilutions), reagent grade, or equivalent, water will be used. The purified water available via faucets (typically labeled DE) at the laboratory sinks, is used for Tecan operation and is also called Tecan system liquid.

4.1 Manual Cycle Sequencing

4.1.1	Dilute BigDye sequencing mix with water.	
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Dilution is based on a calculation of 4.5 µL BigDye + 0.845 µL water per well.

Primer Selection:

For samples amplified with standard primers or mini-primer sets, the primers used for sequencing are different concentrations of the primers used in amplification. Typical primers used in whole control region (WCR) sequencing are A1, B4, A4, 16511, 19, D2, 317, 617. Primer 296 may be used in place of D2 or when needed.

Primers A4 and B4 are used with samples that contain a homopolymeric region between positions 16183 -16194 in the HV1 region (A4 is used with HV1B or HV1, B4 is used with HV1A or HV1). Primers 317 and D2 are used with samples which exhibit significant length heteroplasmy in the HV2 region (317 is used with HV2B or HV2, and D2 is used with HV2).

4.1.2	As necessary, prepare sample and control dilutions using water as the diluent.	
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Based upon the concentrations determined during post amplification quantification, amplified mtDNA template (i.e., sample, HL60) may be diluted to approximately 10 ng per 3.5 μ L. Reagent blanks and negative controls are diluted to the same extent as the sample.

4.1.3	Add reagents/template to the corresponding tubes according to the table below.	
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Manual Cycle Sequencing Reaction Volumes

Component	μ L per well
Diluted BigDye	4.75
1 μ M primer	1.75
Template (i.e., sample, RB)	3.5
Total volume	10

The target amount of mtDNA template is approximately 10 ng per 3.5 μ L, however, lesser or greater amounts may be used as long as the same volume of RB and NC are used as appropriate.

4.1.4	Place tubes in the thermal cycler. Start the "CYCLESEQ" program: <ul style="list-style-type: none"> • 96°C for 1 minute • 25 cycles: <ul style="list-style-type: none"> 96°C for 15 seconds 50°C for 1 second 60°C for 1 minute • 4°C hold indefinitely 	
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Samples are ready for purification.

4.2 Automated Cycle Sequencing

4.2.1	Ensure the Tecan is prepared to run. Prior to daily use: <ul style="list-style-type: none"> • Make ~100 mL of 3% bleach to replace bleach in center trough • Clean the outside of the Tecan tips with 70% isopropyl alcohol • Decontaminate the Tecan work deck with 10% bleach • Run the daily start up script Prior to each run: <ul style="list-style-type: none"> • Check system liquid (i.e., purified water) level and replace/refill the carboy if needed. <i>When a carboy is refilled, it should be allowed to de-gas overnight before use.</i> • Check volume of waste container and empty if needed 	
4.2.2	Prepare set of twelve master mix tubes (tubes 1-12 containing primer A1, B4, A4, 16511, 19, D2, 317, B2, D1, 557, 617, and 617, in this order).	

Each tube of master mix must have a ratio of 50% BigDye : 28.125% water : 21.875% primer. The master mix calculation is based on the reaction volumes per well below and appropriate overage.

NOTE: Master mix tubes should only contain the volume needed for one run of the Tecan, including appropriate overage. Additional volume in the master mix tubes can impact the pipetting accuracy of the Tecan.

4.2.3	Set up Tecan deck: <ul style="list-style-type: none"> • Bleach Rack: Ensure the 3% bleach solution in the center trough was replaced prior to daily use. • Plate Rack: Spin down 96-well plate of purified amplification product and carefully remove seal. Place plate into the rear position of the plate rack. Place an empty 96-well plate in the center position of the plate rack. • Master Mix Rack: Vortex and spin down the twelve tubes of sequencing master mix. Place into positions 1-12 in the order specified in step 4.2.2. 	
4.2.4	Start the cycle sequencing script. The script will prompt the user for: 1) the well in which the first sample to be sequenced is located and 2) the total number of samples to sequence (4-8). The Tecan will automatically set up the requested sequencing reactions.	

Automated Cycle Sequencing Reaction Volumes

Component	µL per well
BigDye	4
1 µM primer	1.75
Template (i.e., sample, RB)	2
Water	2.25
Total volume	10

4.2.5	Seal sequencing plate and place in the thermal cycler. Place an optical compression pad, gold side up, onto the top of the sealed plate and close the lid by pressing the lever down completely. Start “CYCLESEQ” program: <ul style="list-style-type: none"> • 96°C for 1 minute • 25 cycles: <ul style="list-style-type: none"> 96°C for 15 seconds 50°C for 1 second 60°C for 1 minute • 4°C hold indefinitely 	
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Samples are ready for purification.

4.3 Manual Centri-Sep Purification

4.3.1	Remove required number of Centri-Sep strips by cutting through foil. Cut through bottom seal. Place Centri-Sep strips into centermost wells of a disposal plate(s) and remove top foil. Spin 450 X g for 2 minutes. Examine to ensure excess liquid has been removed. Repeat spin if necessary.	
4.3.2	Transfer Centri-Sep strips into a labeled 96-well sequencing plate. Carefully add entire cycle sequencing reaction volume to the Centri-Sep strip well. Place sequencing plate in centrifuge so that strips are in same orientation as in first spin. Spin 450 X g for 2 minutes. Examine to ensure sample has been eluted. Repeat spin if necessary. Discard Centri-Sep strips.	
4.3.3	Place the 96-well plate into vacuum centrifuge and spin to dryness. (Typically 20-45 mins. Do not over dry.)	

Dried samples can be stored frozen. Samples are ready for addition of formamide.

4.4 Automated Centri-Sep Purification

4.4.1	Ensure the Tecan is prepared to run. (Refer to step 4.2.1)	
4.4.2	Remove full set of twelve columns of Centri-Sep strips from box. If desired, cut each column to include the number of samples to be sequenced (i.e. if there are four samples to be sequenced, cut each column so that it contains four wells). Cut through bottom seal.	
4.4.3	Place Centri-Sep strips into disposal plate and remove top foil. Spin at 450 X g for 2 minutes in a flat swing-bucket centrifuge. Examine to ensure excess liquid has been removed. Repeat spin if necessary. Transfer Centri-Sep strips into a labeled 96-well sequencing plate.	

The Sephadex in all Centri-Sep wells must be at the same height and completely level following spin. If not, discard the Centri-Sep strips and repeat procedure.

4.4.4	Set up Tecan deck: <ul style="list-style-type: none"> • Bleach Rack: Ensure the 3% bleach solution in the center trough was replaced prior to daily use. 	
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	<ul style="list-style-type: none"> • Plate Rack: Spin down 96-well cycle sequencing plate and carefully remove seal. Place cycle sequencing plate in the center position of the plate rack. Place Centri-Strip plate in front position of plate rack. 	
4.4.5	Start the Centri-Strip script. The Tecan will automatically transfer the entire plate of sequencing product into the corresponding Centri-Sep strip wells.	
4.4.6	Place sequencing plate in centrifuge and balance plate. Spin 450 X g for 2 minutes. Examine to ensure sample has been eluted. Repeat spin if necessary. Discard Centri-Sep strips.	
4.4.7	Place the 96-well plate into vacuum centrifuge and spin to dryness. (Typically for 30 minutes. Do not over dry.)	

Dried samples can be stored frozen. Samples are ready for addition of formamide.

4.5 Manual Addition of Formamide

4.5.1	Add 10 µL formamide into each well that will be injected. Cover wells with septa strip. Vortex and pulse spin plate.	
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Plate is now ready to be loaded onto the genetic analyzer.

4.6 Automated Addition of Formamide

4.6.1	Ensure the Tecan is prepared to run. (Refer to step 4.2.1)	
4.6.2	Set up Tecan deck: <ul style="list-style-type: none"> • Bleach Rack: Ensure the 3% bleach solution in the center trough was replaced prior to daily use. • Plate Rack: Place dried sequencing plate in center position of the plate rack. • Formamide Rack: Vortex formamide. Place into position 3 of the tube rack. 	
4.6.3	Start the Formamide script. The Tecan will automatically transfer 10 µL of formamide to each well of the sequencing plate.	
4.6.4	Cover wells with 96-well septa. Vortex and pulse spin plate.	

Plate is now ready to be loaded onto the genetic analyzer.

4.7 Sequencing using 3130xl Genetic Analyzer

4.7.1 Reagent Replacement

The buffer and water should be changed daily, when used.

For the first run of the day:

4.7.1.1	Replace the 1X Buffer in anode and cathode buffer reservoirs. Replace the water in water reservoirs.	
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4.7.2 Creating the Plate Record

This may be done at the most convenient time during the procedure.

When creating the Plate Record:

- The plate should be named to include the Laboratory Number, sample(s) or batch number, and biologist initials. *This information may be truncated or abbreviated if needed due to character limitations.*
- Application: "SequencingAnalysis"
- Type: "96-Well plate"

Within the Plate Record:

- Enter the required information (e.g., Sample Name, Results Group) for the applicable wells.
- The laboratory number and date should be entered in Comment field.
- Select "BigDye" for Instrument Protocol 1 and Analysis Protocol 1.

As an alternative, plate records can be created in Excel (or equivalent) and imported as a text (.txt) file onto 3130xl computer.

4.7.3 Loading Plate

At the discretion of the examiner, the sequencing plate may be denatured on a thermal cycler prior to loading or re-injecting using the following method:

Hold at 95°C for 3 minutes

Hold at 4°C for 3 minutes

Hold at 4°C for infinity (∞)

4.7.3.1	Place 96-well plate into a plate base and attach plate retainer/top. Load the plate onto the 3130xl. Ensure the assembly is flat on the autosampler. In the Data Collection "Run Scheduler", link the appropriate Plate Record to the corresponding plate grid. <i>Two plates may be simultaneously run.</i>	
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4.7.3.2	Ensure the doors on the 3130xl are closed and start the run.	
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4.8 Re-Injection of Sequencing Plates

Re-injections (of an entire plate or portions) may be performed by creating or modifying the plate record. Re-injections should include the corresponding positive control in addition to the samples/controls determined by the Examiner.

4.9 Re-Cycle Sequencing

Re-cycle sequencing of a sample must include positive and negative controls. Re-cycle-sequencing of the reagent blank is at the discretion of the Examiner, as necessary for interpretation.

Re-cycle sequencing of samples processed either manually or with automated procedures may be performed with the Examiner's choice of sequencing primer(s). Re-cycle sequencing of samples that were processed with automated procedures will be performed with undiluted template and the reagent amounts detailed in step 4.1.3.

The appropriate controls (i.e., RB, HL60, NC) must accompany any cycle-sequencing using different primers not previously cycle sequenced.

The Examiner may decide to re-amplify or re-extract based upon the sequencing data.

5 Sampling

Not applicable.

6 Calculations

When possible, the target mtDNA template for manual cycle-sequencing is typically 10 ng per 3.5 µL. The mitochondrial DNA case workbook is used to perform and record appropriate dilution calculations, including overage, for the applicable cycle-sequencing reactions. The calculation for determining the volume of amplified sample used in the dilution for the reaction template is based on the post amplification quantification results and the following formula:

$$C_1V_1 = C_2V_2 \rightarrow V_1 = (C_2V_2)/C_1$$

Where C_1 = Quant result (ng/µL)

V_1 = Volume of sample to add to amplification reaction dilution (µL)

C_2 = Target Concentration (i.e., 10 ng/3.5 µl)

V_2 = Maximum input volume (i.e., 3.5 µL per reaction plus appropriate overage)

7 Measurement Uncertainty

Not applicable.

8 Limitations

8.1 Successful sequencing is dependent upon the quantity or quality of DNA in the sample.

9 Safety

9.1 Refer to the “Hazardous Waste Disposal” section of the *FBI Laboratory Safety Manual* for important information concerning proper disposal of the chemicals used in these procedures.

9.2 Procedural Specific Chemical Hazards:

- Formamide is a suspected teratogen. Wear appropriate PPE when handling.
- POP-6 is a chemical hazard and exposure may cause eye, skin and respiratory tract irritation. Wear proper PPE when handling.

10 References

FBI Laboratory Quality Assurance Manual

FBI Laboratory Safety Manual

DNA Procedures Manual

Applied Biosystems. GeneAmp® PCR System 9700 User’s Manual Set. 1997.

Applied Biosystems. 3130/3130xl Genetic Analyzers Getting Started Guide. 2004.

Applied Biosystems. 3130/3130xl Genetic Analyzers Maintenance, Troubleshooting, and Reference Guide. 2004.

Rev. #	Issue Date	History
1	02/28/18	<p>Added guidance about appropriate water to section 4.</p> <p>Added sections 4.2, 4.4, and 4.5 to incorporate automated procedures.</p> <p>Renumbered existing sections.</p> <p>Added option to denature plate to 4.7.3</p> <p>Added Appendix A from mito QC instructions in STACS</p>
2	12/02/19	<p>1 Simplified scope</p> <p>2 Revised to disposal plate, added septa and formamide</p> <p>3 Added requirement for negative control during re-cycle sequencing</p> <p>4.1.2 Removed requirement to record dilutions and this is done by the workbook as described in section 6.</p> <p>4.2.1 Moved Roboscrub procedures to Appendix A</p> <p>4.2.2 Moved and added to master mix guidance below step</p> <p>4.3.3 Reworded italics to match next section header.</p> <p>4.4.1 and 4.6.1 Removed redundant Tecan prep instructions and referenced earlier step.</p> <p>4.6.2 Changed from 8 tubes of formamide to 1</p> <p>4.7 Moved polymer change instructions to QC appendix</p> <p>4.9 Added re-cycle sequencing guidance/instructions</p> <p>5 deleted Sample Selection</p> <p>6 Added target volume calculation</p> <p>Appendix A: Editorial revisions, revised 9700 PV instructions, added action for failed PV, added 3130 weekly maintenance procedures, revised reagent QC, added action for failed QC</p>

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 11/29/2019

DCU Chief

Date: 11/29/2019

Appendix A: Quality Control Procedures

1. Instruments

Refer to the DNA procedure for instrument calibration and maintenance (i.e., DNA QA 608) for minimum frequency of performance verifications (PV) and general maintenance as well as for any additional requirements.

A. Tecan Robotic Workstation

1. Performance Verification of the Tecan Robotic Workstation

Refer to the nuclear DNA quantification procedure (i.e., DNA 226) for instructions for the PV of the Tecan Robotic Workstation.

2. General Maintenance of the Tecan Robotic Workstation

RoboScrub is performed weekly, generally at the end of the workday:

- Make ~3.5 L of diluted Liquinox solution (see instructions on bottle)
- ~3.5 L purified water in a separate container is needed
- Run the RoboScrub Clean script, and follow the prompts

B. Performance Verification of the 9700 Thermal Cycler

Refer to the *GeneAmp*® PCR System 9700 User's Manual Set 96-Well Sample Block Module User's Manual for instructions on how to perform the following procedures.

- **Cleaning** - Refer to *Cleaning the sample wells* and *Cleaning the sample block cover* sections.
- **Temperature Verification Test** - This test procedure requires the use of a Temperature Verification System (Applied Biosystems) and verifies that the thermal cycler remains within the temperature accuracy specification. Refer to *Running the Calibration Verification Test* section.
- **Temperature Non-uniformity Test** - This test procedure requires the use of a Temperature Verification System and verifies the temperature uniformity of the sample wells in the thermal cycler. Refer to *Running the Temperature Non-uniformity Test* section.
- **Rate Test and Cycle Test** - These procedures verify the integrity of the cooling and heating system of a thermal cycler. Refer to *Running System Performance Diagnostics* section.

C. 3130xl Genetic Analyzers

Spatial calibrations, spectral calibrations, and positive control quality control (QC) plates are run as appropriate. New capillary arrays will be installed as needed based on quality of sequence data generated.

1. Performance Verification of the 3130xl Genetic Analyzers

Performance verification (PV) is performed by sequencing 16 HL60 samples with a single primer. High quality sequence data must be obtained from all 16 capillaries. Sequences will be analyzed by the Technical Leader, an mtDNA qualified examiner or DSU personnel with mito data analysis training or experience.

If the expected sequence data is not obtained, the PV will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

Records will be maintained.

2. General Maintenance of the 3130xl Genetic Analyzers

Diagnostic and maintenance procedures are detailed in “Applied Biosystems 3130/3130xl Genetic Analyzers: Getting Started Guide” Chapter 1 (“Preparing the Instrument”), Chapter 2 (“Performing a Spatial Calibration”), Chapter 3 (“Performing a Spectral Calibration”), and Chapter 7 (“Running the Instrument”). The following recommended intervals and instructions provide guidance for the general maintenance of the 3130xl Genetic Analyzer to include instruction for changing the capillary array and performing a spatial calibration or a spectral calibration.

General Maintenance	Recommended Interval
Replace Polymer	Weekly
Replace 1X Buffer/Water/Waste	Before first run of the day and weekly
Install New Array	As needed
Water Wash	When new lot of polymer is used
Flush Water Trap	Weekly
Spatial Calibration	If the array is changed or temporarily removed from the detection block, if the instrument is moved, or as needed
Spectral Calibration	As needed

Weekly Maintenance of the Applied Biosystems 3130xl

The POP-6 should be replaced after 7 days on an instrument. The day that polymer is changed on the instrument counts as day one.

- Prepare a new bottle of POP-6
 - Loosen the POP-6 bottle cap and allow it to sit on the bench top for approximately 15 minutes to degas.
- Selection of the maintenance module to run is dependent upon the lot number of the POP-6 bottles, both new and current:
 - If the new POP-6 bottle is the same lot number as the current bottle, run the **Replenish Polymer Wizard**
 - If the new POP-6 bottle is a different lot number compared to the current bottle, run the **Water Wash Wizard**
 1. Flush the polymer delivery pump (PDP)
 2. Run the **Water Wash Wizard** and use reagent grade water to flush the PDP.
Note: For a warm water wash, heat water to < 60°C.
 3. Follow wizard prompts.
- Flush the PDP water trap
 - Use a 20 mL syringe filled with reagent grade water.
 - Attach the syringe to the forward facing fitting at the top of the pump block, open the fitting approximately one-half turn counter clockwise.
 - Open the exit fitting at the top left side of the pump block approximately one half turn counter clockwise.
 - Flush the water trap with approximately 5 mL of water.
 - Close both fittings by turning them clockwise until finger-tight, do not over tighten.

Replacing POP-6 outside of the regular maintenance schedule:

- Allow the POP-6 to equilibrate to room temperature and degas.
- Run the **Replenish Polymer Wizard** in the 3130xl software.
- Flush the water trap in the upper polymer block.
- Note the date of POP-6 replacement and record the reagent lot numbers.

Replacing a capillary array:

Arrays will be replaced as needed based on data quality or when visibly broken and leaking polymer.

- Close the oven and instrument doors, and then press the Tray button.
- Select Wizards > **Install Array Wizard**.

The capillary array length in the wizard must match the length being used (36 or 50 cm).

- Open instrument and oven doors.
- Follow the directions in the wizard to install or replace array.
- Close and lock the oven door. Close the instrument doors.

Performing a spatial calibration:

Spatial calibration is required after installing or replacing a capillary array, temporarily removing the capillary array from the detection block, or moving the instrument.

- In Data Collection, click Spatial Run Scheduler.
- If the capillaries contain fresh polymer, select Protocol > 3130SpatialNoFill_1. Otherwise, select Protocol> 3130SpatialFill_1.
- Click Start. *A spatial calibration run lasts approximately two minutes without filling the capillaries and six minutes when filling the capillaries. The spatial profile window turns black when you initiate a calibration run. Peaks will be drawn in as the spatial proceeds.*
- Evaluate the spatial calibration profile using the following criteria:
 - Similar heights for all peaks
 - Single sharp peak for each capillary (small shoulders will be acceptable)
 - Single orange cross marking the top of every peak
 - Spacing of 13 to 16 pixels between each peak
- If the calibration passes, click “Accept” to write the calibration data to the database.
- If the calibration fails, click “Reject”. If split peaks are the cause of failure, remove the array window and wash with a small amount of methanol, then dry and re-run spatial. Repeat as necessary.

Performing a spectral calibration:

- Remove a tube of BigDye Terminator v1.1 Sequencing Standard from the freezer. Add 170 µL of formamide to resuspend. Vortex thoroughly. Briefly centrifuge the mixture.
- Add 10 µL of the diluted Sequencing Standard to wells A1 through H2 of a 96-well reaction plate.
- Place septa over the wells and heat the plate in a thermal cycler at 95°C for 5 minutes to denature the DNA.
- Cool the plate in a freezer for two minutes. Briefly centrifuge the plate.
- Place the sample plate into a plate base. Snap the plate retainer onto the base.
- In Data Collection, click Plate Manager. Click New to open the New Plate dialog box. Enter a name for the plate. In the Application drop-down list, select Spectral Calibration. In the Plate Type, select 96-Well. Enter initials for the owner and plate operator. Click OK. Enter sample names and comments. In the Instrument Protocol 1 column, select the BigDye sequencing standard spectral protocol from the list (usually named bdv1.1_seq_spectral or similar).
- Place the plate assembly on the autosampler and link the plate record. Begin the run. *A spectral run will take approximately fifty minutes to complete.*
- After the run, the pass/fail status of each capillary will be displayed in the Spectral Viewer. View the status of each capillary by clicking on the pictorial representation of the plate. Passing capillaries will be:

- Colored green
- Have a Q-value above 0.95
- Have a condition number range between 3 and 5
- A passing spectral run will automatically become the active calibration for the BigDye v1.1 dye set, replacing the previously run spectral for that dye set.
- A spectral run with failed capillaries will be re-run until all capillaries pass.

2. Critical Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., DNA QA 609) for additional requirements.

A. Sequencing Reagents

1. Sequencing primers will be tested by amplifying a positive (HL60) and a negative control sample and cycle sequencing with the appropriate sequencing primers used for that amplified region.
2. BigDye Sequencing Kits will be tested by sequencing whole control region (WCR) amplified product (32 cycles) of a positive (HL60) and a negative control sample with A1 and 617 primers only.

Multiple positive control samples may be amplified; at least one must yield data and result in the expected positive control sequence.

If the expected positive control sequence is not obtained, the QC will be repeated. If the results are still deemed unsuitable, the Technical Leader will be consulted.

Procedures for mtDNA Sequence Analysis, Interpretation, and Report Writing

1 Scope

These procedures describe the use of the software packages for mitochondrial DNA (mtDNA) sequence analysis and assembly, as well as how to perform mtDNA comparisons, interpretations, weight assessments, and write reports.

2 Equipment/Material/Reagents

Sequencing Analysis Software (Applied Biosystems™, version 6.0 or higher)

Sequencher™ Software (Gene Codes Corporation, version 5.0 or higher)

PopStats (CODIS, version 7.0 or higher)

EMPOP (Available at <http://empop.online/>, current version)

3 Procedure for Analysis of Data Using Sequencing Analysis

Raw sequencing data is first processed using Sequencing Analysis to trim data obtained by the sequencer before and after the actual sequence. This allows for maximum quality of the sequencing data.

The steps outlined below describe the general steps involved in analyzing the data from sequencing runs. Alternative pathways or shortcuts for accomplishing the same tasks may be used.

1. Launch Sequencing Analysis software. A username and password are required.
2. Add files to the Sample Manager window by selecting “Add Sample” from the File menu. Select desired run folder and choose desired analysis files. Click “Add Selected Samples” and “OK.”
3. To view data from a sample file, click to check the “Show” box or highlight all samples and click on the “Show” button. The electropherogram may be viewed by clicking on the “Electropherogram” tab.
4. Display the raw data by clicking on the “Raw” tab. Note the beginning and ending scan numbers by moving the cursor to the beginning and end of data peaks (the scan numbers should be similar for all capillaries within a run). Signal intensity may also be reviewed in the “Raw” tab.

5. Enter the beginning scan numbers in the “Peak 1” and “Start” columns. This ensures that the first sequenced base is included.
6. Enter the ending scan numbers in the “Stop” column. This will decrease the number of “Ns” at the end of the run and focus the signal intensity calculations on the appropriate section. All runs [NC (Negative Control), RB (Reagent Blank), sample, and HL60 (positive control)] with a given primer must have the same “Peak 1” “Start” and “Stop” values. Note: A single “Peak 1” and “Start,” as well as a single “Stop” number may be used for all files within a run, as long as these numbers encompass all sequence data in each file.
7. Ensure the “BC” (BaseCaller) column is checked.
8. Click on the green arrow button to start the analysis.
9. Review the resulting electropherograms in the “Electropherogram” tab to ensure the applicable range of sequence data has been captured. Save the files. **Note: The saved files still retain all of the raw data.**

4 Procedure for Assembly of Sequences Using Sequencher™

Trimmed sequence data is assembled and edited using Sequencher™. Two strands (one from the forward primer and one from the reverse primer) must be used to edit sequence whenever possible. Single-stranded sequence can be used, but can be only edited toward ambiguity (the most conservative editing call) when there is a surrounding context for making the editing decision. Artificial insertions, deletions, or ambiguities created by the Sequencing Analysis software may be edited in single-stranded sequence if supported by the surrounding context.

The steps outlined below describe the general steps involved in assembling the data from various primers to yield an mtDNA sequence. Alternative pathways or shortcuts for accomplishing the same tasks may be used.

1. Launch Sequencher™ software, using the settings represented in Appendix A.
2. Open “New Project” under the “File” pull-down menu.
3. Highlight the files you wish to import and drag them into the project window. Open each sequence individually and delete unanalyzable sequence from the ends. Note which samples are not analyzable (typically this includes RBs and NCs).
4. Highlight analyzable forward and reverse sequences for assembly and click on “Assemble Automatically.” The default settings of 85 minimum match percentage and 20 minimum overlap are usually sufficient for assembly of samples and positive controls

(see Appendix A, Figure 1). Generally, RBs and NCs that do not assemble under the default parameters should be considered not analyzable.

5. View the contig produced. Check the direction of the sequence files by looking at the primers in the file name. If they are not in the appropriate orientation, go to the view menu and select “Reverse Comp.”
6. Click on the “Bases” box to observe the nucleotide sequence and click on “Show Chromatograms” to observe the electropherograms. Delete the amplification primer sequences.
7. Edit the sequences using information from all strands in the electropherogram window until the forward and reverse primer sequences are the same at all positions. Each base in the sequence must be manually reviewed for correct identification using the electropherogram windows. It is possible that ambiguous bases will remain after the sequence is fully edited. These ambiguities will be designated according to International Union of Pure and Applied Chemistry (IUPAC) nomenclature (see Section 5 for more details).

If the sequence is not of requisite quality (e.g., high background, poor peak resolution, or indicative of a mixture), the sequence will not be used. The sample may be re-extracted, re-amplified, re-cycle sequenced, or re-injected, as appropriate.

A mixture is a sequence with multiple mixed base positions. Determination of a mixture is dependent on the length of sequence obtained, the contextual sequence information, and the site of the mixed base positions. If any region of a sample is determined to be a mixture, absent primer binding site mutations, the entire extract will be considered a mixture and not used. If available, the sample will be re-extracted.

8. When the editing is completed, import the revised Cambridge Reference Sequence (rCRS) into the project. Highlight the rCRS and the contig(s) relevant to the sample (e.g., HV1 and HV2) in the project window and click on “assemble automatically.” A new contig will be created which will consist of the sample’s consensus sequence and the rCRS.

Note: The rCRS may be imported and assembled with the sample sequences in Step 4 above. At a minimum, the contig is named to reflect the sample designation and the appropriate symbols/initials. Ensure base calls adhere to the sequence nomenclature set forth in Section 5.

9. Differences from the rCRS can be viewed in the contig window or by highlighting the contig and choosing “Compare Consensus to Reference” from the “Contig” pull-down menu.
10. The Difference file can be saved by exporting it as “Report Format: Individual Reports.”

Ensure the exported file and/or the Sequencher™ project view contain(s) the following information: Laboratory Number, Examiner symbols/initials, sample ID, date, and sequence range(s). On the Sequencher™ project view, sequences which are not analyzable are noted “NA”, and sequences which are analyzable but do not align with the rCRS are noted “ABNA.”

5 Sequence Nomenclature

- Polymorphisms are noted on the Sequencher™ files and transferred to a sequence summary sheet by noting the nucleotide position followed by the code for the polymorphic base (e.g., 263 G). Nucleotide base positions are designated according to the standard nomenclature set by IUPAC and listed in the table below:

CODE	DESIGNATION
A	Adenine
B	C, G, or T
C	Cytosine
D	A, G, or T
G	Guanine
H	A, C, or T
K	G or T (Keto)
M	A or C (aMino)
N	aNy base or combination of bases
R	A or G (puRine)
S	G or C
T	Thymine
V	A, C, or G
W	A or T
Y	C or T (pYrimidine)
OTHER	DESIGNATION
-	Deletion
X.1(.2,...)	Insertion after position X

- Variants from the rCRS will be coded in accordance with the hierarchical nomenclature rules from *SWGDAM Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories* (reproduced text below in italics):

Rule 1 – *Maintain known patterns of polymorphisms (a.k.a. known phylogenetic alignments). Most violations to known patterns of polymorphisms involve insertions and deletions.*

Insertions are described by noting the site immediately prior to the insertion followed by a “.1” (for the first insertion), a “.2” (if there is a second insertion), and so on, and then by the nucleotide that is inserted (e.g., 315.1 C). Deletions are noted by a dash (-).

All sequences containing insertions or deletions outside the expected HV1, HV2, or HV3 C-stretch regions, as well as nucleotide positions 249, 290, 291, and between 514 - 525, will be compared to known phylogenetic alignments.

For assistance with identifying known patterns of polymorphisms, the sequence may be compared to those listed in the *SWGDAM Mitochondrial DNA Nomenclature Examples Document*, queried using an approved phylogenetic alignment tool, or a subject matter expert (SME), as designated by the Technical Leader, may be consulted.

Rule 2 – *Use nomenclature with the least number of differences.*

Rule 3a – *Homopolymeric C-stretches in Hypervariable Region 1 (HV1): C-stretches in HV1 should be interpreted with a 16189C when the otherwise anchored T at position 16189 is not present. Length variation in the short A-tract preceding 16184 should be noted as transversions.*

Rule 3b – *Homopolymeric C-stretches in Hypervariable Region 2 (HV2): C-stretches in HV2 will be named with a 310 C when the otherwise anchored T at position 310 is not present.*

When a 310 C occurs, no insertions relative to the rCRS at 303-315 are noted.

Rule 4 – *Maintain the AC repeat motif in the HVIII region, generally between np 514-525.*

The AC repeat is treated as a single unit, with indels grouped immediately 5' to np 525. A single AC insertion is noted as 524.1 A, 524.2 C and a deletion of AC is noted as 523 -, 524 -. Length variants will not be recorded for this region, but the sequence will be trimmed, if needed, to include all bases which do not exhibit other underlying bases.

Rule 5 – *Prefer substitutions to insertions/deletions (indels).*

Rule 6 – *Prefer transitions to transversions.*

Rule 7 – *Place indels contiguously when possible.*

Rule 8 – *Place indels on the 3' end of the light strand.*

3. Point or sequence heteroplasmy is defined as more than one base at a given position (independent of overlap of peaks created by length variants), present in both sequencing directions and above the level of background noise. Typically, point heteroplasmy is detected at only one or two positions within the mtDNA control region for a given sample.
4. Length heteroplasmy is defined as more than one length variant in a given region and typically occurs in the homopolymeric C stretch region in HV1, HV2, and/or HV3.

Length heteroplasmy in HV1 most commonly arises when there is a substitution of a C for a T at position 16189. The reference type in HV1 is C₅TC₄. HV1 length variants will not be recorded in casework samples due to the pronounced out-of-phase sequence following this stretch of Cs. Generally, the sequences will be truncated to fit the format of the reference. This is accomplished by starting at position 16180 in the forward strand and truncating after the 14th base. The reverse strand is truncated by counting backwards starting at position 16193 and matching the number of Cs in the forward strand. Only non-C insertions will be recorded following position 16193 when length heteroplasmy is observed in this area.

Length variants in HV2 are commonly observed in the number of C residues preceding a T residue at position 310. The reference type in HV2 is C₇TC₅. Length variants of C residues between positions 302 - 310 will not be noted. Instead, the major length variant as called by Sequencher™ will be included in the Difference file and listed in a sequence summary table.

Length variants in HV3 will not be noted and typically will be based on single-stranded data. The sequence will be trimmed to include all bases which do not exhibit other underlying bases.

5. Typically, the range is defined as the span of double-stranded sequence obtained and denoted by the positions relative to the rCRS. In some instances, single-stranded data at the 5' or 3' end or surrounding C-stretch regions may be included in the range.

6 Procedure for Sequence Comparisons

6.1 Evaluation of Control Sequences

The positive control must type correctly (see Section 9) and be of requisite quality in order to report the sequence for an associated sample.

After RB and NC sequence data are analyzed, a determination is made as to whether any analyzable sequence is present. If analyzable sequence is present in any RB or NC, it must first be compared to the sequence obtained from the associated sample to determine whether the contamination seen in the RB or NC could be the source of the sequence seen in the sample. To

eliminate this possibility, the sequence of the sample and the corresponding RB or NC must differ at one or more base positions within each amplified region. A difference in length variants is not considered as a base difference when comparing RB and NC sequences against the sample sequence. If the sequence of the RB or NC within a region is concordant with the sample, the sample should be either re-extracted, re-amplified, or re-cycle sequenced as appropriate at the Examiner's discretion. If these methods fail to resolve the issue, the region may not be included in the reported sequence for that sample.

6.2 Comparisons Among Samples

When comparing sequences obtained from samples, only the regions with a common range will be evaluated. For example, if a partial sequence (16024-16365 and 73-284) is obtained for a sample of unknown origin, and a full sequence is obtained for the sample of known origin, the comparison will be conducted on positions 16024-16365 and 73-284 only. Cytosine insertions at nucleotide position 309 will not be used for comparison purposes.

6.3 Interpretation of Sequence Comparisons

The following conclusions are determined for sequence comparisons:

Exclusion:	If the samples differ at two or more positions, they are excluded as coming from the same source.
Inconclusive:	If the samples differ at a single position only the comparison is inconclusive.

Because of the possibility of undetected heteroplasmy, additional samples of known origin may be analyzed when sequences from samples of unknown origin and sequences from samples of known origin differ at a single position. Known origin samples may include blood, buccal swabs, and hair. Hair fragments from a hair standard of known origin may be combined and processed as a single known sample.

Cannot Exclude:	If the samples have the same sequence, or demonstrate sequence concordance, they cannot be excluded as potentially coming from the same source. Sequence concordance is defined as having a common base at each position at which sequence data were obtained in the sample.
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6.4 Sequence Confirmation

The sequences for all samples and associated controls (HL60, NC, and RB) must be confirmed by a second qualified individual. Confirmation of the sample, NC, and RB involves independently evaluating and assembling all of the same sequence data from the analysis data that were used by the reporting Examiner. Confirmed sequence range is defined as the shortest length of sequence upon which the Examiner and the confirmer have reached consensus. Separate Sequencher™ projects and Difference files are generated by each individual.

The confirmer will evaluate the assembled and edited HL60 contig (i.e., set of overlapping DNA segments) for the sample and indicate approval by appending the Difference file name with their symbols/initials.

Confirmation of mixtures or of samples of insufficient quality will be indicated by annotation of the confirmer's Sequencher™ project with “mixture” or “insufficient quality.” Portions of samples not used in the final project due to insufficient data quality do not need to be reviewed by a confirmer.

For cases in which the interpretation “Cannot Exclude” has been made, proceed to Section 7, “Procedure for Weight Assessment.” For other cases, proceed to Section 8, “Procedure for Report Writing.”

7 Procedure for Weight Assessment

7.1 Searching Profiles in CODIS

Typically, a CODIS Popstats search is conducted when samples of unknown origin and samples of known origin are found to be concordant. CODIS 7.0 SP3 or higher is used to search casework profiles in the database using the settings represented in Appendix B.

Those database samples whose range(s) are fully included within the casework profile range are used in the database search result. Presently, this includes nucleotide positions 16024-576.

Ambiguous base positions are searched using the appropriate IUPAC symbol (see section 5).

The CODIS Popstats database search result provides the number of database profiles that match the casework profile, as well as the number of profiles in the database that differ by up to five positions. In addition, the search result details the database profiles that have 0 base differences from the search profile, which may be included in the case file. Special attention should be paid to database search results from queries with sequences with a 310 C, as well as with non-C insertions following position 16193.

7.2 Special Considerations

All sequence polymorphisms in the sample searched are entered; however, all length variants at nucleotide positions 16193, 309, and 573 are ignored in database searches of relevant concordant sequences. Hence, length variability in these regions will not add any additional rarity to a database profile search.

7.3 Upper Bound Frequency Estimate

The one-tailed Clopper and Pearson method for determining the upper bound frequency estimate of a mtDNA profile is used at a confidence level of 95% ($\alpha = 0.05$).

The following formulae are used:

In cases where the profile has been observed in the database:

$$\sum_{k=0}^x \binom{n}{k} p_0^k (1 - p_0)^{n-k} = \alpha$$

This equation finds the value p_0 of the population proportion p for which the cumulative probability 0, 1, . . . x copies of the profile is equal to α and n is the number of profiles in that population. This equation will require a computer to solve.

In cases where the profile has not been observed in the database ($x=0$):

$$p_0 = 1 - \alpha^{1/n}$$

7.4 Searching Profiles in EMPOP

In order to provide additional information regarding population groups relevant to a case, but not contained within the CODIS mtDNA population database, a search may be conducted using the EMPOP database (<http://www.empop.online>), with search parameters set to “pattern” match type, and disregarded insertions/deletions (indels) at positions 16193, 309, and 573 (see Appendix C). Note that EMPOP is compatible with the Firefox internet browser, but not Internet Explorer.

The EMPOP search result is provided for geographic origin affiliations: Africa, America, Asia, and Europe. Relevant subgroups within these populations can be selected in the “Find origin” box. The number of observations and number of profiles searched is entered into a table in the report for the EMPOP search (see Section 8.1.15). If a one-tailed Clopper and Pearson frequency estimate is not provided in the EMPOP search, it will be calculated using the formula in Section 7.3, and the value entered into the report table.

8 Procedure for Report Writing

The following examples for wording of Results of Examination, Methodology and Interpretation, and Remarks sections are meant to represent typical case situations. Determination of the exact wording is made by the Examiner of record on a case-by-case basis.

8.1 Results of Examinations Section

As appropriate, the final sentence in the Results of Examinations section will be “No other mtDNA examinations were conducted.”

8.1.1 Cannot Exclude (Full Sequence)

For concordant samples of unknown origin and known origin, the Report of Examination will include a table (see below) containing the CODIS 7.0 SP3 Popstats (or higher) search results of relevant population groups, to include at least the African-American, US Caucasian, and US Hispanic groups. Any group in which there is an observation of the sequence will also be included in the table. Population groups in which the searched profile was not observed are noted in a footnote to the table (see footnote below table, with population sample size indicated parenthetically). When multiple database searches have been conducted in a case and have the same results, a single table may be incorporated into the report.

“Mitochondrial DNA (mtDNA) sequences were obtained from item 1 and DOE. The mtDNA sequences obtained from item 1 and DOE are the same within the sequence range obtained in common to both samples. Therefore, DOE cannot be excluded as the source of item 1.

Searching the CODIS mtDNA population database (CODIS 7.0 SP3, containing 10,629 individuals, searching positions 16024-16390 and 49-408)^A, the mtDNA sequence obtained from item 1 and DOE has been observed as follows^B:

Population Group	Number of Observations	Number of Profiles in Population Group	Upper Bound Frequency Estimate
African-American	0	2449	0.12%
US Caucasian	0	2609	0.11%
US Hispanic	0	2576	0.12%

In addition to the population groups listed in the table above, the population database search included a search of mtDNA sequences from individuals from China – unspecified region (168), China – Hong Kong (376), Japan (302), Korea (281), US Asian (645), Vietnam (187), US Native American (1036) with no observation of the queried sequence in these groups. The numbers in parentheses indicate the number of individuals in each population group.

^A The range of sequence positions included in a database search is the one obtained in common for the samples.

^B The population database table(s) above has been included to indicate how common or rare a

sequence is expected to be in the general population. The upper bound frequency estimate is based on a 95% confidence interval and gives an estimate of the highest percentage of individuals in each population group expected to have the same profile as the referenced samples. Calculation of the upper bound frequency estimate is directly dependent upon the number of profiles in the population group; larger population group sizes will provide more refined upper bound frequency estimates. Mitochondrial DNA profiles were placed into population groups within the database based on self-identification by the donor. A searched profile may or may not appear in the population database or it may be observed within multiple groups in the database. Therefore, mtDNA cannot be used to identify the population group to which an individual belongs.”

8.1.2 Cannot Exclude (Partial Sequence)

“A mitochondrial DNA (mtDNA) sequence was obtained from DOE. A partial mitochondrial DNA (mtDNA) sequence was obtained from item 1 (nucleotide positions 16005-16236 and 49-408 only). The mtDNA sequences obtained from item 1 and DOE are the same across the sequence range obtained in common to both samples. Therefore, DOE cannot be excluded as the source of item 1.

Searching the CODIS mtDNA population database (CODIS 7.0 SP3, containing 10,629 individuals, searching positions 16024-16236 and 73-340)^A, the mtDNA sequence obtained from item 1 and DOE has been observed as follows^B:”

Include the database search results table and explanatory wording as in 8.1.1.

Note: A limited sequence range will only be indicated in the report if it is less than the minimum range of HV1 and HV2 (i.e., less than 16024-16365 and 73-340).

8.1.3 Concordance (Sequence Heteroplasmy)

“Mitochondrial DNA (mtDNA) sequences were obtained from item 1 and DOE. The mtDNA sequence obtained from item 1 is the same as the mtDNA sequence obtained from DOE, with the exception of position 16069. At this position, the presence of a thymine (T) was observed in item 1. In DOE, evidence of both a thymine (T) and a cytosine (C) was characterized at position 16069. Since the sequences obtained from item 1 and DOE are concordant, JOHN DOE cannot be excluded as the source of the item 1 hair.

Searching the CODIS mtDNA population database (CODIS 7.0 SP3, containing 10,629 individuals, searching positions 16024-16365 and 73-340)^A, the mtDNA sequence obtained from item 1 and DOE (including both nucleotides, C and T, at position 16069) has been observed as follows^B:”

Include the database search results table and explanatory wording as in 8.1.1.

8.1.4 Exclusion

“Mitochondrial DNA (mtDNA) sequences were obtained from item 1 and DOE. The mtDNA sequence obtained from item 1 is different than the sequence obtained from DOE. Therefore, DOE is excluded as the source of item 1.”

8.1.5 Inconclusive (One Base Difference)

“Mitochondrial DNA (mtDNA) sequences were obtained from item 1 and DOE. The mtDNA sequence obtained from item 1 is the same as that obtained from DOE, with the exception of position 16069. At this position, the presence of a thymine (T) was observed in item 1. In DOE, a cytosine (C) was characterized at position 16069.

Due to the one base pair difference observed between item 1 and DOE, no conclusion could be reached as to whether DOE can be excluded as the source of item 1.”

8.1.6 Evidentiary Samples Concordant

“Mitochondrial DNA (mtDNA) sequences were obtained from items 1 and 2. The mtDNA sequences obtained from items 1 and 2 are the same. Therefore, items 1 and 2 cannot be excluded as originating from a common source.”

A CODIS Popstats search will be conducted in such cases.

8.1.7 Mixture

“The mitochondrial DNA (mtDNA) sequence obtained from item 1 indicates the presence of a mixture of mtDNA from more than one individual. Because mixtures of mtDNA are not interpretable, no comparisons can be performed using item 1.”

8.1.8 No Sequence Obtained

“There was insufficient mitochondrial DNA (mtDNA) present for a DNA sequence to be obtained from items 1 or 2.”

8.1.9 Not of Requisite Quality:

“The mitochondrial DNA (mtDNA) sequence obtained from item 1 was not of the requisite quality for comparisons or interpretations to be conducted.”

8.1.10 Missing Person Reference Sample

“A mitochondrial DNA (mtDNA) sequence was obtained from MARY DOE, the mother of JANE DOE (NCIC No. MXXXXXXXXX). The mtDNA profile from MARY DOE will be entered into the Relatives of Missing Persons Index of the Combined DNA Index System (CODIS) and maintained by the FBI Laboratory for future comparisons.”

8.1.11 Missing Person Unknown Origin Sample

“A mitochondrial DNA (mtDNA) sequence was obtained from item 1 (NCIC No. UXXXXXXXXX). The mtDNA profile from item 1 will be entered into the Unidentified Human (Remains) Index of the Combined DNA Index System (CODIS) and maintained by the FBI Laboratory for future comparisons.”

8.1.12 Matching mtDNA Sequences from a CODIS search

“This report supplements the FBI Laboratory report dated September 15, 2008. Refer to that original report for the results of the previous mtDNA testing. This report contains the results of a CODIS (Combined DNA Index System) search.”

Results of the CODIS search:

“As a result of searching the FBI's CODIS database, the mtDNA profile obtained from item 1 (ID: AB/CD010101001Q1) has the same mtDNA profile as a biological sibling of JANE DOE (ODTL-02-0299) submitted by the [name of other DNA testing laboratory]. Therefore, JANE DOE cannot be excluded as the source of item 1 (bone).”

8.1.13 TEDAC Cases

“A mitochondrial DNA (mtDNA) sequence was obtained from item 1 (CEXC XX/XXX/XX) and will be entered and searched in the appropriate databases. This profile is retained for future comparisons.”

8.1.14 Cannot Exclude using an EMPOP search

Typically, a CODIS Popstats search will be conducted along with an EMPOP search. Therefore, the wording from Section 8.1 would be used in addition to the following (Note: The number of subpopulation groups, as well as the number of individuals searched, is case dependent):

“A search of the European DNA Profiling Group Mitochondrial DNA Population Database (EMPOP; currently available online at <http://empop.org>) was also performed to provide additional information regarding population groups relevant to this case.

Searching the EMPOP population database^C (EMPOP version 11, containing 27,479 individuals within the searched sequence range of positions 16024-16390 and 49-408)^D the mtDNA sequence obtained from items 1 and 2 has been observed as follows^E:

Geographic Affiliation	Number of Observations	Number of Individuals in Population Group	Upper Bound Frequency Estimate
AFRICA	0	1900	0.16%
AMERICA	0	14813	0.02%
ASIA	1	6440	0.07%
South East Asia			
Philippines	1	153	3.06%
Indonesia	0	277	1.08%
Malaysia	0	123	2.41%
Thailand	0	190	1.56%
Vietnam	0	187	1.59%
East Asia			
China	0	544	0.55%
Japan	0	402	0.74%
Korea	0	692	0.43%
EUROPE	0	4326	0.07%

^C The EMPOP search was performed with "pattern" match type search parameters and disregarded indels at positions 16193, 309, and 573.

^D The range of sequence positions included in a database search is the one obtained in common for the samples.

^E The CODIS and EMPOP population database tables(s) above have been included to indicate how common or rare a sequence is expected to be in the general population. The upper bound frequency estimate is based on a 95% confidence interval and gives an estimate of the highest percentage of individuals in each population group expected to have the same profile as items 1 and 2. Calculation of the upper bound frequency estimate is directly dependent upon the number of profiles in the population group; larger population group sizes will provide more refined upper bound frequency estimates. Mitochondrial DNA profiles were placed into population groups within the CODIS database (first table) based on self-identification by the donor, and within the EMPOP database (second table) based on geographic affiliation. Some population groups within the CODIS database contain a subset of profiles from similar groups contained within EMPOP. A searched profile may or may not appear in the population database or it may be observed within multiple groups in the database. Therefore, mtDNA cannot be used to identify the population or subpopulation group to which an individual belongs.”

8.2 Methodology and Interpretation Section

8.2.1 If a Comparison is Conducted

“DNA is extracted from each sample, and portions of the control region of the mitochondrial DNA are amplified using the polymerase chain reaction (PCR). The amplified regions are sequenced using fluorescent dye-labeled chemistry. The sequences obtained are aligned and compared to the rCRS. Differences between the sample sequence and the rCRS are noted by nucleotide position and DNA base. The annotated profiles for all of the samples are then compared. Matching profiles may be searched against the CODIS mtDNA population database to provide an upper bound frequency estimate.

Mitochondrial DNA cannot be used to conclusively identify an individual because mtDNA is maternally inherited and all maternally-related individuals are expected to have the same mtDNA profile. Also, unrelated individuals may have the same mtDNA profile within the sequenced range.

The following interpretations are possible for sequence comparisons:

CANNOT EXCLUDE:

If samples have the same sequence, or are concordant (share a common DNA base at every nucleotide position), they cannot be excluded as coming from the same source or maternal lineage.

INCONCLUSIVE:

If sample sequences differ at a single nucleotide position no conclusion can be reached as to whether they originate from the same source.

EXCLUSION:

If sample sequences differ at two or more nucleotide positions they are excluded as coming from the same source.

When an EMPOP search is conducted, the final sentence of the first paragraph may be modified, as appropriate:

“Matching profiles may be searched against the CODIS mtDNA population database as well as the EMPOP mtDNA population database to provide an upper bound frequency estimate.”

8.2.2 If No Comparison is Conducted

“DNA is extracted from each sample and portions of the control region of the mitochondrial DNA are amplified using the polymerase chain reaction (PCR). The amplified regions are sequenced using fluorescent dye-labeled chemistry. The sequences obtained are aligned and compared to the rCRS. Differences between the sample sequence and the rCRS are noted by nucleotide position and DNA base.

Mitochondrial DNA cannot be used to conclusively identify an individual because mtDNA is maternally inherited and all maternally-related individuals are expected to have the same mtDNA profile. Also, unrelated individuals may have the same mtDNA profile within the sequenced range.”

8.3 Remarks Section

As appropriate, the Remarks section of the report may include one or more statements similar to those below:

8.3.1 Standard Remarks Section

“This report contains the opinions/interpretations of the examiner(s) who issued the report. The supporting records for the opinions and interpretations expressed in this report are retained in the FBI files. The submitted items will be returned to you under separate cover. For questions about the content of this report, please contact Forensic Examiner Jane Doe at (insert phone number).”

“In addition to the evidence in the case, the DNA Casework Unit has generated secondary evidence that will also be returned to you. The secondary evidence can be found in a package marked DNA Casework Unit Secondary Evidence. It is recommended that this evidence be stored in a refrigerator or freezer and isolated from evidence that has not been examined.”

8.3.2 Consumption of Evidence

“It is noted that item 1 was consumed during the DNA Casework Unit examinations.”

8.3.3 Missing Person Cases

“Mitochondrial DNA profiles from unidentified human remains that are uploaded to the National DNA Index System (NDIS) require a complete mtDNA profile (at a minimum, nucleotide positions 16024-16365 and 73-340). If uploading to NDIS is required, additional skeletal material is needed. Please submit more skeletal material such as a femur, tibia, or other whole bone sample if available.”

“A portion of item 4 was retained in the National Missing Person DNA Database Repository for possible future testing. Archive samples will be retained by the FBI Laboratory until an association is made. Archive samples will be returned to the contributor if requested. Archive samples that are not requested to be returned within 60 days of the report date will be used anonymously for training and validation studies.”

8.3.4 Return of Archival Sample

“On February 28, 2011 Dr. Ben Jones notified Examiner John Doe by telephone and facsimile that the UNKNOWN FEMALE DECEASED has been identified by dental examination. The

return of all skeletal material was requested and the DNA profiles that were placed into the Combined DNA Index System (CODIS) will be removed. The portion of item 1 that was retained for archive will be returned under separate cover.”

8.3.5 TEDAC Cases

“The supporting records for the opinions and interpretations expressed in this report are retained in the FBI files. For questions about the content of this report, please contact mtDNA Examiner Jane Doe at (insert phone number).”

8.3.6 No mtDNA Examination - Remains Non-Human Origin

“The item 1 bone was examined by Dr. Jane Smith, Curator, Department of Anthropology, Smithsonian Institution, Washington, DC. Dr. Smith's report is enclosed herewith. Because the bone is not of human origin, no mitochondrial DNA examinations were conducted.”

8.3.7 Requests Not Conducted

“The DNA typing results obtained from the tested items are not eligible for entry into the Combined DNA Index System (CODIS).”

8.3.8 CODIS Association Reports

“The information provided by the University of North Texas Center for Human Identification for the missing person is:

Redacted

Please contact Lt. Jane Smith of the University of Virginia Police Department at (insert phone number) or jsmith@virginia.edu for potential investigative information.

A portion of Item 1 was retained for archival purposes for possible future testing. Archive samples will be retained by the FBI Laboratory until an association is made. Archive samples

will be returned to the contributor if requested. Archive samples that are not requested to be returned within 60 days of the date of this report may be used anonymously for training and validation studies.

It is noted that the results of the nuclear DNA CODIS association will be the subject of a separate report. Please contact the DNA Casework Unit at **Redacted** for further information regarding the nuclear DNA association.

The supporting records for the opinions and interpretations expressed in this report are retained in the FBI files. For questions or to advise the FBI Laboratory of any identifications made based on the content of this report, please contact mtDNA Examiner Jane Doe at (insert phone number).

This information is provided only as an investigative lead.”

9 Standards and Controls

The controls used throughout mtDNA analysis are the RB, NC, and HL60. Refer to Section 6.1 for the interpretation of these controls.

9.1 Reagent Blanks and Negative Controls

1. The RB monitors for the presence of exogenous DNA from the extraction steps through sequencing and is processed from the DNA extraction procedure through the sequencing procedure. If a sample is re-extracted, a new RB is generated in the repeated extraction.
2. The NC monitors for the presence of exogenous DNA from the amplification procedure through the sequencing procedure.
3. If a sample is re-amplified with the same primers, sample volume, and amplification parameters (cycle #), the associated RB does not need to be re-amplified, provided that an HL60 and NC are included with the re-amplification.

If a sample, RB, and/or NC requires re-cycle sequencing and/or re-injection, the HL60 will also be re-cycle sequenced and/or re-injected. If only the HL60 requires re-cycle sequencing and/or re-injection, only the HL60 needs to be processed.

4. Regardless of the relative quantity of mtDNA in the RB, NC, and the sample, if the RB or NC sequence is in concordance with the sample, the results of the amplicon are not used for comparison purposes. The sample sequence can be used under the following conditions:

- a) the RB or NC does not analyze;
- b) the RB or NC analyzes but is not of requisite quality for comparison purposes;
- c) the RB or NC sequence analyzes, but the sequence is not in concordance with the sample

5. If analyzable mtDNA sequence is obtained from the RB or NC, an attempt is made to determine the possible source of this DNA.

9.2 Positive Control

1. The use of a positive control monitors the success of amplification, sequencing, and sequence analysis. The HL60 cell line DNA is used as the positive control for mtDNA analysis.
2. The mtDNA analysis of a particular sample extract is repeated, going back to the appropriate step as judged by the Examiner, if the positive control fails to amplify (e.g., is <1ng/μl) and/or sequence correctly.

9.2.1 Positive Control Sequences

HL60 DNA is used as a positive control. The whole control region (WCR) of the mtDNA from this cell line has been amplified and sequenced by the FBI Laboratory. The HL60 mtDNA WCR sequence, expressed as differences from the rCRS, is provided in the table below. The mtDNA HV1 and HV2 sequences for the cell lines 9947A and K562 are provided for information.

Cell Line	HL60 (WCR)	9947A	K562
HV1	16069 T 16193 T 16278 T 16362 C	16311 C	16126 C 16294 T 16296 T 16324 C
HV2	73 G 150 T 152 C 263 G 295 T 315.1 C 489 C (outside of HV2)	93 G 195 C 214 G 263 G 309.1 C 309.2 C 315.1 C	73 G 263 G 310-313 D

10 Sampling

Not applicable.

11 Calculations

A 95% confidence interval is calculated by the CODIS Popstats software in order to determine an upper bound frequency estimate of individuals within each population who are estimated to be included as potential contributors of a particular profile, as described in Section 7.3.

12 Measurement Uncertainty

Not applicable.

13 Limitations

Mitochondrial DNA analysis is subject to limitations defined in the extensive validation studies conducted by FBI scientists prior to the implementation of the procedure. Publications describing this validation and subsequent studies are listed in Section 17.

13.1 Because of the sensitivity of mtDNA analysis, contamination is a concern. To this end, measures are taken to minimize and monitor any DNA foreign to the sample. To remove contaminating DNA present on the sample prior to extraction, samples are cleaned with detergents, bleach, and/or other chemicals whenever possible. In some instances, this cleaning may not remove all of the contaminant and a mixed mtDNA profile may result. Extensive quality control procedures (e.g., cleaning of work surfaces and equipment with bleach and/or ultraviolet light prior to use, assessing all critical reagents prior to use on casework samples, physical separation of pre-amplification and post-amplification work areas) are employed to minimize the introduction of foreign DNA into the sample. In addition, RBs and NCs monitor for contamination introduced during the analytical processes. Although the optimal outcome is for no mtDNA to be detected in these controls, controls with sequence the same as that of the associated sample can yield partial or no interpretable results for that sample.

13.2 Although mtDNA analysis is a sensitive technique, it is possible that the quantity of DNA present in some samples is too low to yield results with the current mtDNA methodologies.

13.3 Mitochondrial DNA is maternally inherited and is not unique to an individual. All maternal relatives are expected to have the same mtDNA sequence, absent mutation. In addition, unrelated individuals may share the same mtDNA sequence.

13.4 Mixtures of mtDNA from more than one individual are not interpretable and are reported as such.

14 Safety

Not applicable.

15 Sequences of Amplification and Sequencing Primers

Primers used only for sequencing are marked with an asterisk (*).

Standard Primers

A1	(L 15997)	5'-CAC CAT TAG CAC CCA AAG CT-3'
B2	(H 16237)	5'-GGC TTT GGA GTT GCA GTT GAT-3'
A2	(L 16159)	5'-TAC TTG ACC ACC TGT AGT AC-3'
B1	(H 16391)	5'-GAG GAT GGT GGT CAA GGG AC-3'
A4*	(L 16209)	5'-CCC CAT GCT TAC AAG CAA GT-3'
B4*	(H 16164)	5'-TTT GAT GTG GAT TGG GTT T-3'
C1	(L 048)	5'-CTC ACG GGA GCT CTC CAT GC-3'
D2	(H 285)	5'-GGG GTT TGG TGG AAA TTT TTT G-3'
C2	(L 177)	5'-TTA TTT ATC GCA CCT ACG TTC AAT-3'
D1	(H 409)	5'-CTG TTA AAA GTG CAT ACC GCC-3'
317*	(L 317)	5'-CCC CCC CTC CCC CCG C-3'

Whole Control Region Primers

A1	(L 15997)	5'-CAC CAT TAG CAC CCA AAG CT-3'
B4*	(H 16164)	5'-TTT GAT GTG GAT TGG GTT T-3'
A4*	(L 16209)	5'-CCC CAT GCT TAC AAG CAA GT-3'
16511*	(L 16511)	5'-CCG ACA TCT GGT TCC TAC-3'
19*	(H 19)	5'-TCC CGT GAG TGG TTA ATA G-3'
296*	(H 296)	5'-RRR GGG GGG GTT TGG TG-3'
317*	(L317)	5'-CCC CCC CTC CCC CCG C-3'
617	(H 617)	5'-TGA TGT GAG CCC GTC TAA AC-3'
557*	(H 557)	5'-GGG GGG TGT CTT TGG GG-3'

Mini-Primers

1A	(L 16055) 5'-GAA GCA GAT TTG GGT ACC AC-3'
1B	(H 16142) 5'-ATG TAC TAC AGG TGG TCA AG-3'
2A	(L 16131) 5'-CAC CAT GAA TAT TGT ACG GT-3'
2B	(H 16218) 5'-TGT GTG ATA GTT GAG GGT TG-3'
3A	(L 16209) 5'-CCC CAT GCT TAC AAG CAA GT-3'
3B	(H 16303) 5'-TGG CTT TAT GTA CTA TGT AC-3'
4A	(L 16287) 5'-CAC TAG GAT ACC AAC AAA CC-3'
4B	(H 16356) 5'-GTC ATC CAT GGG GAC GAG AA-3'
5A	(L 16347) 5'-CGT ACA TAG CAC ATT ACA GT-3'
5B	(H 16410) 5'-GCG GGA TAT TGA TTT CAC GG-3'

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Rev. #	Issue Date	History
2	04/22/14	Unit names removed or changed throughout as appropriate. Changed evidence examples to reflect FA naming throughout. Grammatical changes made throughout. Clarified RB/NC wording in Section 5.1. Revised wording in Sections 3.3, 5.1, and 8.1 to remove restrictions on rework as allowed by the QAS. Deleted previous Section 6.1 and renumbered. Added instructions for using EMPOP searches for weight assessment in Section 6 and wording for such searches in Section 7.1.15 and 7.2.1. Modified wording for use of CODIS in reports in Sections 7.1.1, 7.1.2, 7.1.3, and 7.2.1. Simplified example in Section 7.1.14. Clarified 10% threshold wording in Section 14.1.
3	04/21/16	Grammatical changes made throughout. Updated and Relocated Equipment/Material/Reagents section to 2 and renumbered remaining. Appendices A, B, and C created and referred to in document where appropriate. Substituted parameter setting wording for Appendix A and removed changing parameters for RB and NC alignment in Section 4.4. Revised wording for annotation of electronic files instead of printed documents in Sections 4.10, 5.1 and 6.4. Clarified nomenclature wording and included requirement for obtaining phylogenetic guidance in Section 5.2. Removed requirement for recording HV2 length variants in Section 5.4. Clarified wording in Section 6.1. Removed Section 6.2.1. Removed use of length variants and insertions following 309 for comparison purposes in Sections 6.2 and 6.3. Removed redundant wording in Section 7.1. Reworded to reflect current practices in Sections 7.1 and 7.2. Specified use of a one-tailed statistic in Section 7.3. Revised to reflect current EMPOP settings and browser compatibility in Section 7.4. Updated to reflect current report wording and incorporate changes due to not recording length variants or insertions following 309 for comparison purposes in Section 8. Removed Section 8.1.6 and renumbered following sections. Added requirement for Popstats search in reports with concordant evidentiary samples in current Section 8.1.6. Removed reference to 10% rule in Section 9.1. Updated Sections 10, 11, and 13. Moved Pos Control Sequence into 9 and renumbered remaining. Added appropriate references to Section 16.

Approval

 Redacted - Signatures on File

Appendix A: *Sequencher Settings*

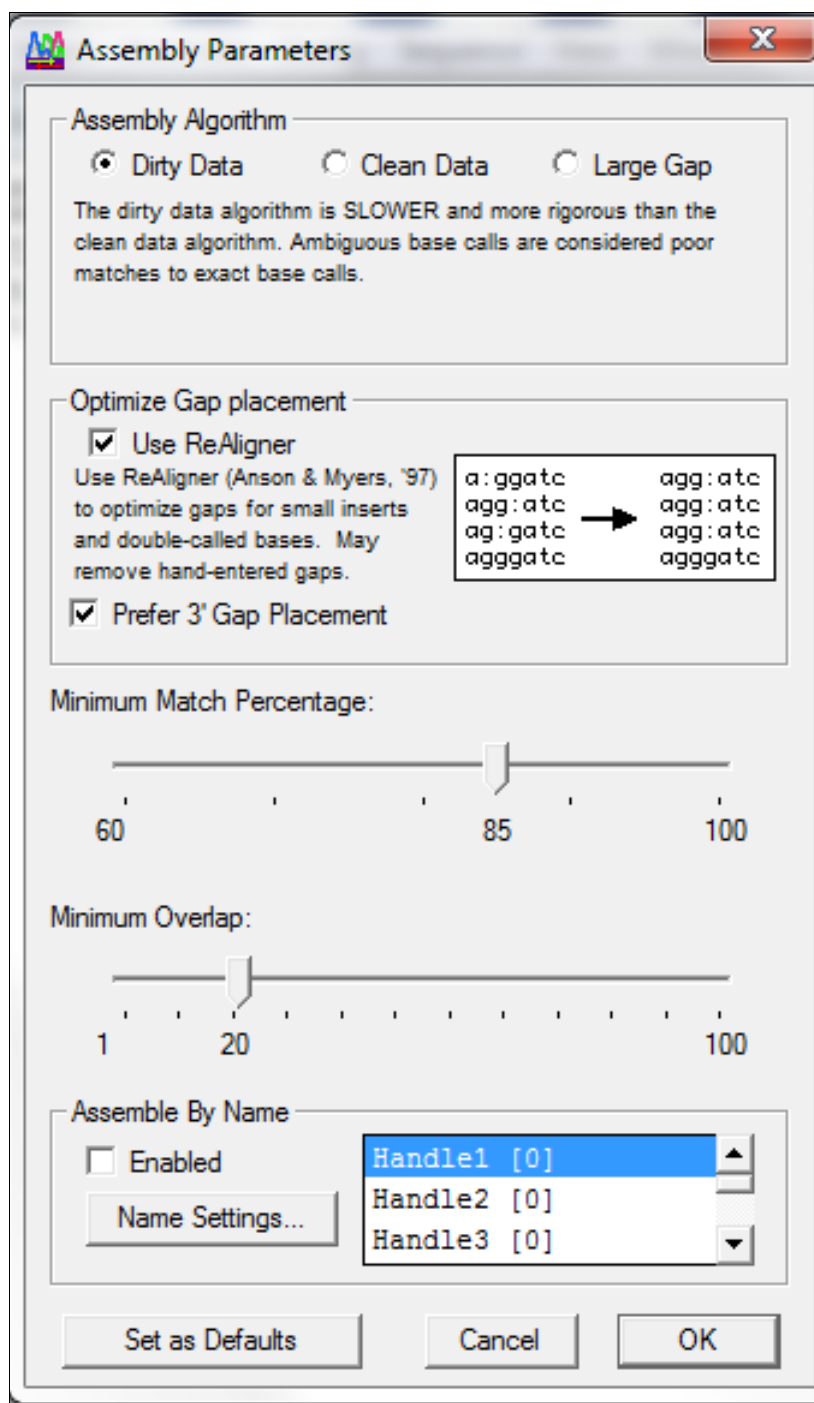


Figure 1 – Assembly Parameters

Appendix A: *Sequencher Settings (cont.)*

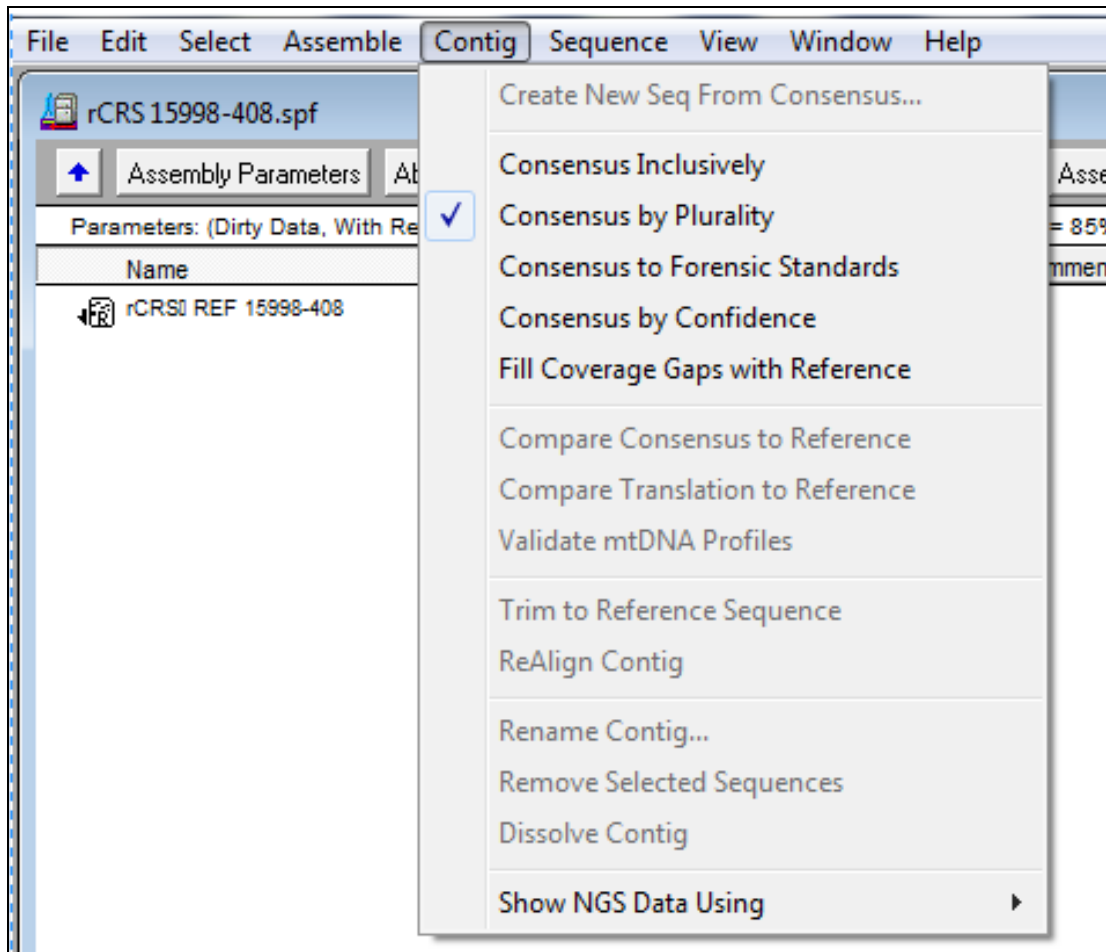


Figure 2 – Contig Consensus

Appendix A: *Sequencher Settings (cont.)*

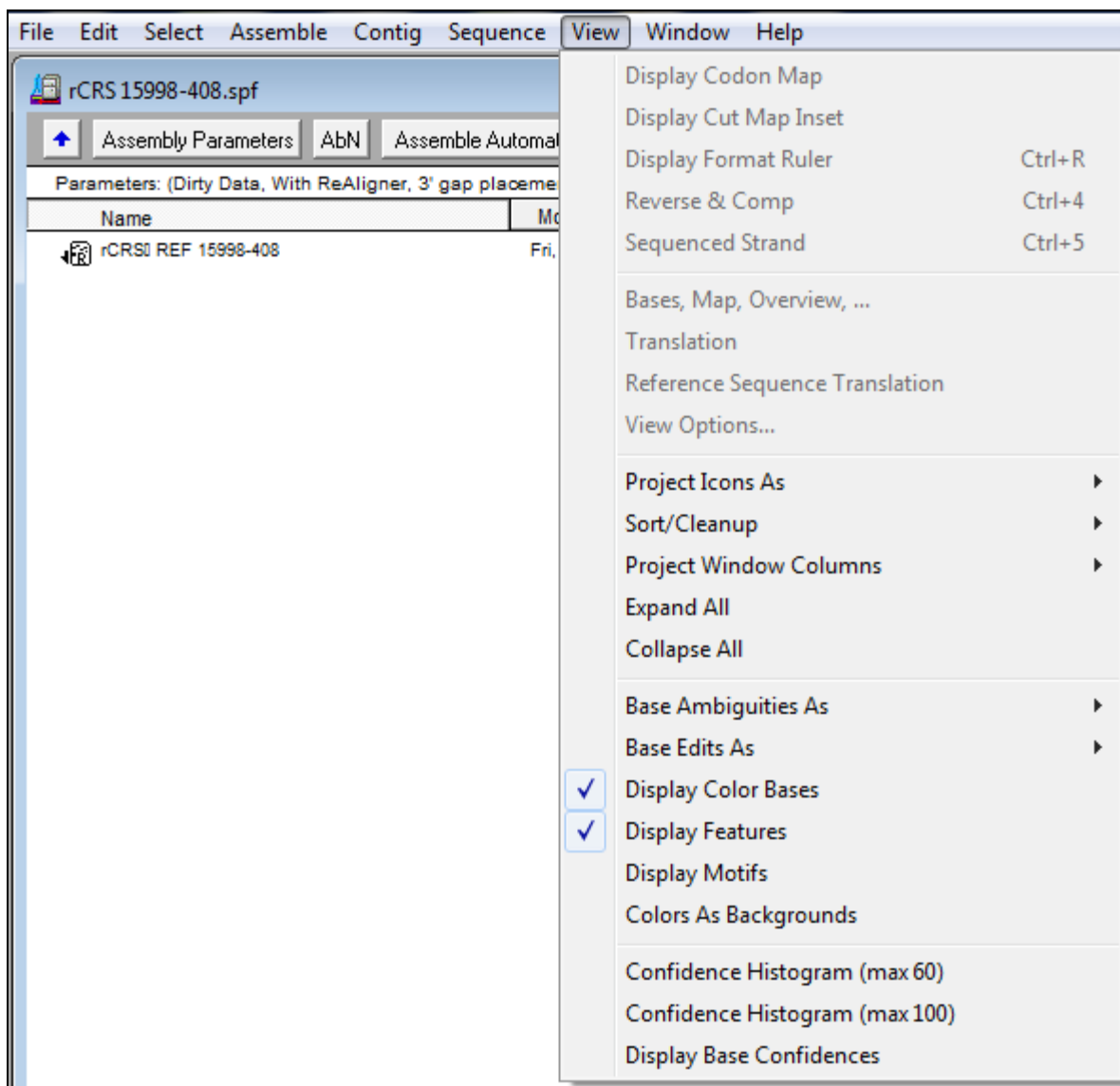


Figure 3 – View settings

Appendix A: *Sequencher Settings (cont.)*

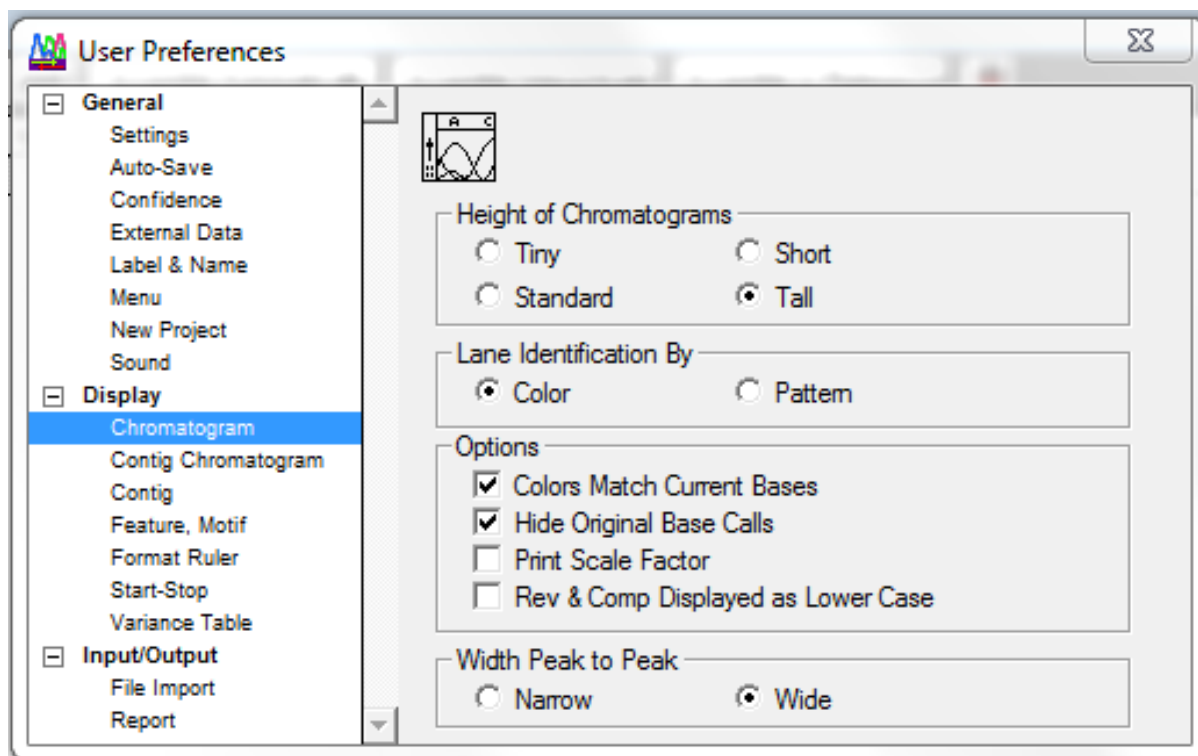


Figure 4 – User Preferences

Appendix B: Analyst Workbench – CODIS Popstats

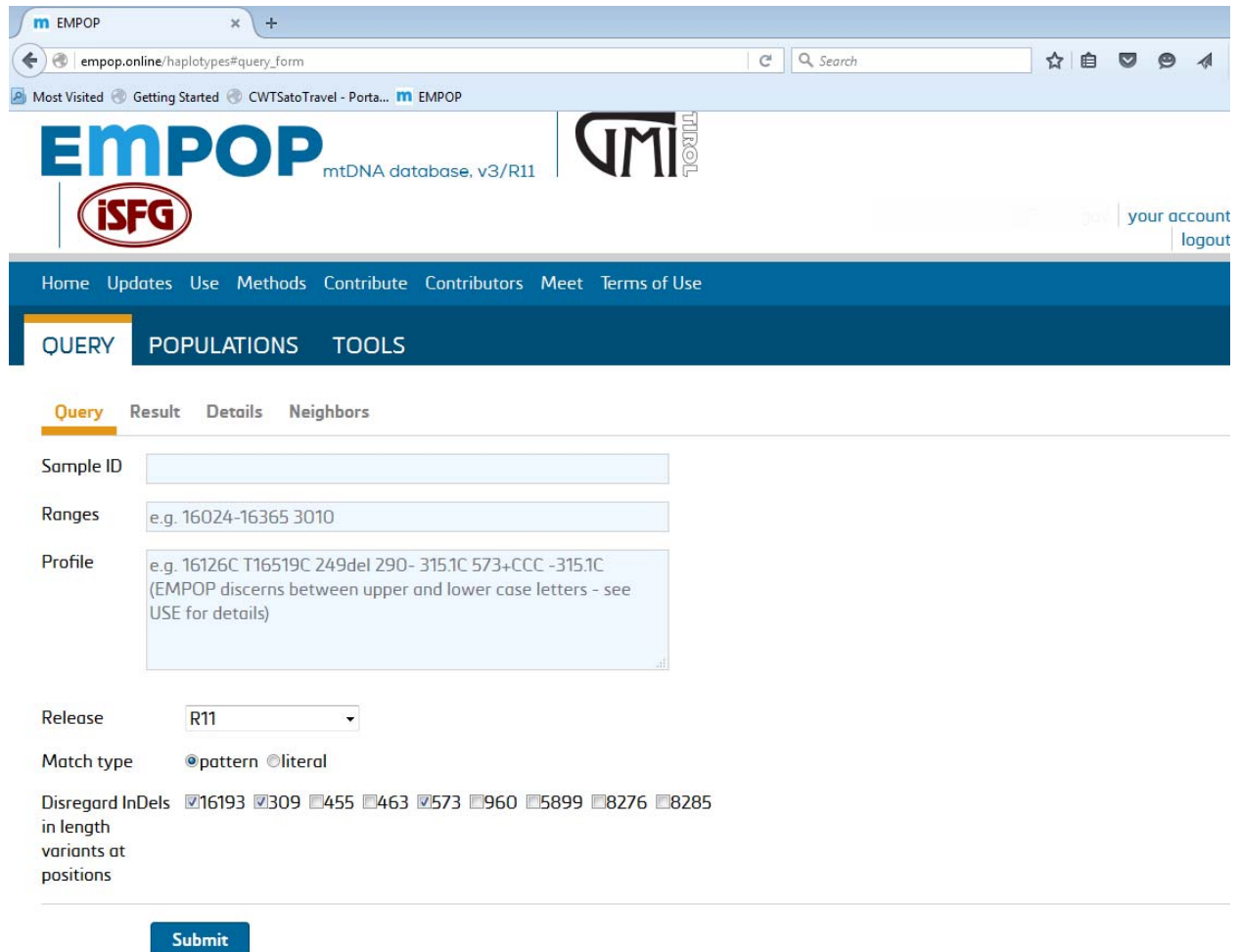
The screenshot displays the 'CODIS Popstats' dialog box with the following settings:

- Database Information:**
 - Name: mtDNA_Population_Data
 - Description: Parson W and Dür A (2007) EMPOP-a forensic mtDNA database. FSI:Genetics 1(2), 88-92; Scientific Working Group on DNA Analysis Methods (SWGDAM) Interpretation Guidelines for Mitochondrial DNA Analysis by Forensic DNA Testing Laboratories, July 2013. Please refer to the CODIS 7.0 SP3 release notes for further information regarding the mtDNA population database.
- Population Groups to Search:**
 - ☒ African Origin
 - ☒ Caucasian Origin
 - ☒ Hispanic Origin
 - ☒ Asian Origin
 - ☒ Native Origin
- Confidence Interval (95%) Method:**
 - ☒ Clopper-Pearson One-Tailed
 - ☐ Normal Approximation Two-Tailed
- Length Heteroplasmy:**
 - 309
 - 573
 - 16193
- Match Parameters:**
 - Maximum number of sequence differences for match: 5
 - Maximum number of sequence differences for display: 0
 - Minimum number of overlapping base pairs for search: 50
 - Minimum pop. group size for upper bound frequency estimate: 150
 - List the match pairs ☒
- Length heteroplasmy option:**
 - ☒ Ignore insertions at the specified length heteroplasmy sites
 - ☐ Consider insertions at the specified length heteroplasmy sites as individual differences

Buttons: Apply, OK, Cancel

Default settings for CODIS mtDNA population search

Appendix C: EMPOP



The screenshot shows the EMPOP website interface. The browser address bar displays "empop.online/haplotypes#query_form". The website header includes the EMPOP logo (mtDNA database, v3/R11), the ISFG logo, and the University of Tübingen logo. A navigation bar contains links: Home, Updates, Use, Methods, Contribute, Contributors, Meet, Terms of Use. Below this is a secondary navigation bar with "QUERY", "POPULATIONS", and "TOOLS". The "QUERY" tab is active, showing sub-tabs: Query, Result, Details, Neighbors. The "Query" sub-tab is selected, displaying a form with the following fields and options:

- Sample ID**: A text input field.
- Ranges**: A text input field with the example "e.g. 16024-16365 3010".
- Profile**: A text input field with the example "e.g. 16126C T16519C 249del 290- 315.1C 573+CCC -315.1C (EMPOP discerns between upper and lower case letters - see USE for details)".
- Release**: A dropdown menu set to "R11".
- Match type**: Radio buttons for "pattern" (selected) and "literal".
- Disregard InDels in length variants at positions**: A row of checkboxes for specific positions: 16193 (checked), 309 (checked), 455 (unchecked), 463 (unchecked), 573 (checked), 960 (unchecked), 5899 (unchecked), 8276 (unchecked), 8285 (unchecked).

A blue "Submit" button is located at the bottom of the form.

Figure 1 - Settings for EMPOP database search

Appendix C: EMPOP (con't)

EMPOP mtDNA database, v3/R11

ISFG

UMI

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QUERY POPULATIONS TOOLS

[Query](#) **[Result](#)** [Details](#) [Neighbors](#)

Sample ID **2016-00000**
Ranges 16024-576
Profile 263G 315.1C

Entire Database	Frequency	Clopper Pearson CI	estimate p
	58/26127	2.2199e-3	[1.6861e-3, 2.8688e-3]

By Origin	Frequency	Clopper Pearson CI
Africa	10/1900	5.2632e-3 [2.5267e-3, 9.6578e-3]
America	18/13829	1.3016e-3 [7.7159e-4, 2.0563e-3]
Asia	6/6024	9.9602e-4 [3.6561e-4, 2.1666e-3]
Europe	24/4374	5.4870e-3 [3.5187e-3, 8.1532e-3]

Figure 2 - EMPOP Search Results Screen

DNA Evidence Management Procedures

1 Purpose

This document describes the operational guidance and procedures for evidence management in the DNA Casework Unit (DCU) and the Biometrics Analysis Unit (BAU) DNA Group. The Case Administration Group (CAG) functions as the evidence hub within DCU.

2 Scope

This document applies to DNA personnel performing evidence management tasks, including receiving, transferring, and returning evidence, for DCU and BAU.

3 Responsibilities

Unit personnel with responsibilities related to the assignment of DCU and BAU cases and evidence transfer will follow the responsibilities identified in the relevant practices of the FBI *Laboratory Operations Manual* (LOM).

3.1 CAG personnel will:

- Follow the practices and procedures in the QAM, LOM and the *DNA Procedures Manual* as it pertains to receipt, check-in, inventory, transfer, return, and storage of physical evidence.
- Record all evidence transfers in the appropriate Laboratory Information Management System (LIMS) or on a paper *Chain-of-Custody Log* (7-243/7243a).

3.2 BAU personnel will:

- Follow the practices and procedures in the QAM, LOM, and the *DNA Procedures Manual* as it pertains to receipt, check-in, inventory, transfer, return, and storage of physical evidence.
- Record all evidence transfers in the appropriate Laboratory Information Management System (LIMS), on a paper *Chain-of-Custody Log* (7-243/7-243a) or in the Chain-of-Custody Log in the

4 Procedures

It is noted that the procedures listed below will typically occur in the order described; however, to maximize the workflow, they can occur in a different order, as necessary.

4.1 General Operations

4.1.1 Evidence transfers to/from entities outside of DCU are generally performed by CAG personnel, with the exception of transfers that occur outside of normal Laboratory Division (LD) business hours. In these situations, other appropriately trained personnel may temporarily support CAG functions. Evidence transfers to/from entities outside of BAU are performed by BAU DNA personnel.

4.1.2 All evidence transfers, including secondary evidence transfers, will be recorded in Forensic Advantage (FA), in the Sample Tracking and Control System (STACS), on a paper *Chain-of-Custody Log* (7-243/7-243a), or in the Chain-of-Custody Log in **Redacted** as appropriate, prior to performing any work on the evidence.

4.1.2.1 A virtual transfer is a recording of a transfer within FA, STACS, or **Redacted** where the physical location of the item does not change. Virtual transfers are typically used when switching between the LIMS systems, for administrative reasons (e.g., Chain-of-Custody (COC) corrections in STACS), or due to limitations of the software.

4.1.3 Transfers of drug and valuable evidence will be performed according to the appropriate LOM practice. Appropriate locations for the storage of drug and valuable evidence may be physically located outside of DCU or BAU controlled space; however, virtual storage locations for these areas will be available within STACS.

4.1.4 Evidence will be stored under appropriate conditions. In general, liquid blood, tissue, vaginal aspirates, and DNA extracts will be refrigerated. Potential latent items should be stored at room temperature. All other evidence may be stored refrigerated, frozen, or at room temperature.

4.1.5 FA, STACS, and/or **Redacted** barcodes/labels may be used, as appropriate, for identifying evidence.

4.1.6 For Office of Professional Responsibility (OPR) investigations or prohibited cases that are conducted outside of FA, the case and sample information entered into STACS will be limited to generic descriptions.

4.2 Evidence Receipt

4.2.1 The listing of incoming evidence placed in the Evidence Storage Room (ESR) can be viewed in the Evidence module of FA. For evidence submitted in Legacy cases, CAG personnel will be notified, typically by email, by the Evidence Management Group (EMG) or the delivering LD unit; BAU personnel will be notified, typically by email, by the Evidence Management Unit (EMU) or the delivering LD unit.

4.2.2 Evidence may be physically transferred in bulk from the DCU ESR to a laboratory space for receiving into DCU by any CAG member as long as the evidence transfers are appropriately recorded. The CAG laboratory space (e.g., Rm 3390) may be used as a temporary storage location during evidence receipt into DCU. Evidence may be physically transferred in bulk from the BAU ESR to a laboratory space for receiving into BAU by any BAU DNA personnel.

4.2.3 Evidence Receipt in a Multiple Unit Submission (MUS)

For legacy cases use the appropriate Chain-of Custody Log in lieu of FA.

4.2.3.1 Retrieve the evidence and record the transfer in FA.

4.2.3.2 Record a virtual evidence transfer in FA by selecting the “Placed in Storage” transfer type and the “DCU (see DCU STACS COC)” or “BAU (see BAU STACS COC)” (or equivalent) storage location.

4.2.3.3 Proceed to Receipt and Inventory in STACS.

4.2.4 Evidence Receipt in a Single Unit Submission (SUS)

4.2.4.1 Retrieve the evidence and record the transfer in FA.

4.2.4.2 Review the EC or incoming communication to determine relevant information regarding the submitted evidence.

4.2.4.3 Perform evidence breakdown according to the appropriate LOM practice.

4.2.4.4 Scan and/or upload appropriate records (e.g., Check-in notes, missing person consent forms) to the Case Object Repository.

4.2.4.5 Record a virtual evidence transfer in FA by selecting the “Placed in Storage” transfer type and the “DCU (see DCU STACS COC)” or “BAU (see BAU STACS COC)” (or equivalent) storage location.

4.2.5 Receipt and Inventory in STACS

4.2.5.1 Use the Receipt and Inventory module to add an evidence container. Scan a STACS container barcode (e.g., AXXXXXE) and apply to the evidence container.

4.2.5.2 Fill out the appropriate information fields pertaining to the evidence container (i.e., Container Description, Seal State Description, and External Unit of origin).

4.2.5.3 Based on the batch details in FA and/or the container markings, select the appropriate items of evidence and drag into the newly created container. Highlight the container, then complete and confirm the container inventory. Repeat, as needed, for additional evidence containers.

4.2.6 Completion of Evidence Receipt

4.2.6.1 As necessary, notify the contributor that the evidence was received within DCU or BAU via telephone call, voicemail, or email and record the contact in the Case Communication Log within FA or STACS. Emails are attached to the Case Communication Log.

4.2.6.2 If a paper case file is generated, print the appropriate FA records (i.e., Case Report and Case Record Report) as well as the appropriate records from the Object Repository (e.g., EC/incoming communication, Sexual Assault Kit (SAK) paperwork, missing persons consent forms, TEDAC submission forms). For a legacy case, the equivalent paperwork may be received or compiled.

4.2.6.3 Use the appropriate transfer module in STACS to transfer the evidence to a person or a storage location.

4.3 Evidence Return

4.3.1 When ensuring all items are accounted for, FA must be referenced for packaging information when the contents are paper based evidence items (e.g., envelopes, letters), with the exception of legacy cases. Packaging expectations for other types of evidence may be obtained from either FA or STACS.

4.3.2 When returning secondary evidence, create a new FA item as needed, and generate a secondary evidence list report in STACS. This secondary evidence log will be uploaded to the FA Case Object Repository for DCU cases and the FA Case Record Object Repository for BAU cases. For legacy cases, the secondary evidence log will be retained with the case records. If secondary evidence from both a MUS and a SUS for a case will be returned at the same time and to the same location, all secondary evidence may be returned with the MUS.

4.3.3 Evidence Return in a Multiple Unit Submission (MUS) and TEDAC cases

For legacy cases use the appropriate Chain-of Custody Log in lieu of FA.

4.3.3.1 Retrieve the evidence and record the transfer in STACS.

4.3.3.2 For evidence containers opened in DCU or BAU, ensure all items are accounted for and sealed appropriately.

4.3.3.3 In the “Transfer to/from External Unit” module in STACS, record a virtual transfer of the evidence by selecting “Transfer to/from FA” (or equivalent) as the external unit or another location, as appropriate.

4.3.3.4 In FA, record and physically transfer the evidence to the appropriate storage location or person.

4.3.4 Evidence Return in a Single Unit Submission (SUS)

For a TEDAC SUS refer to section 4.3.3.

4.3.4.1 Retrieve the evidence and record the transfer in STACS.

4.3.4.2 Ensure all items are accounted for and sealed appropriately.

4.3.4.3 In the “Transfer to/from External Unit” module in STACS, record a virtual transfer of the evidence by selecting “Transfer to/from FA” (or equivalent) as the external unit or another location, as appropriate.

4.3.4.4 Record a virtual evidence transfer in FA from the “DCU (see DCU STACS COC)” or “BAU (see BAU STACS COC)” (or equivalent) storage location to the appropriate person. Package the evidence for return according to the appropriate LOM practice. Ensure the FA label is affixed to the shipping container and the appropriate items within the shipping container are reflected in FA.

4.3.4.5 Ensure the shipping address is correct and generate a *Shipping Invoice* (7-264/7-264 LIMS) in accordance with the appropriate LOM practice.

4.3.4.6 Ensure the *Shipping Invoice* and secondary evidence log (if applicable) are in the shipping container and a copy of the first page of the *Shipping Invoice* is attached to the outside of the container.

4.3.4.7 Physically transfer the sealed shipping container to the mail room and record a hand-to-hand transfer in FA. Sealed shipping containers may be appropriately stored prior to transfer to the mail room.

5 Safety

All laboratory operations will be performed in a safe manner and in accordance with the standards established by applicable regulatory agencies. Personnel in CAG and BAU will follow the health and safety measures outlined in the QAM, *FBI Laboratory Safety Manual*, and the applicable section(s) of relevant DNA procedures.

6 References

FBI Laboratory Quality Assurance Manual

FBI Laboratory Operations Manual

FBI Laboratory Safety Manual

DNA Procedures Manual

Rev. #	Issue Date	History
6	07/17/17	Document discontinued between 02/10/2014 and 07/17/2017. Revised entirety of document to reflect current procedures for evidence receipt and return to include interactions with FA and STACS.
7	10/02/17	Added BAU and legacy throughout. 4.1.7 To addressed OPR cases within STACS

Approval

FBI Approved Standards for Scientific Testimony and Report Language for Serological Examinations

1 Purpose

This document provides examples of statements that are approved for reporting scientifically supported conclusions and offering expert opinions during testimony by Forensic Examiners within the FBI Laboratory's DNA Casework Unit (DCU) and Biometrics Analysis Unit (BAU) DNA Group. It is noted that these examples are not intended to be all inclusive and may be dependent upon the precedent set by the judge or locality in which a testimony is provided. Further, these examples are not intended to serve as precedent for other forensic laboratories and do not imply that statements by other forensic laboratories are incorrect, indefensible, or erroneous.

2 Scope

This document applies to Forensic Examiners who prepare an FBI *Laboratory Report* (7-1, 7-1 LIMS, 7-273, or 7-273 LIMS) and/or provide testimony related to serological examinations.

3 Responsibilities

3.1 The Examiner will ensure that a *Laboratory Report* is consistent with the approved language contained within this document.

3.2 The Examiner will ensure that his/her testimony related to serological examinations is consistent with the standards contained within this document.

3.3 The Technical Reviewer will ensure that a *Laboratory Report* contains language consistent with the standards contained within this document.

3.4 An authorized evaluator will assess if testimony provided by DNA personnel complies with the statements contained within this document in accordance with the FBI Laboratory Operations Manual (LOM) *Practices for Testimony Related Activities*.

4 Statements Approved for FBI Serological Examination Testimony and/or Laboratory Reports

The results of presumptive and/or confirmatory serological tests are used in conjunction to develop a conclusion. The reported conclusion is based on the most informative results in the following order: 1) a positive confirmatory test result, 2) a positive presumptive test result, 3) an inconclusive result, or 4) a negative result. For example, if a presumptive test is positive and a

confirmatory test is negative or inconclusive, the reported conclusion is based on the positive presumptive test.

4.1 Identification of Blood or Semen

The Examiner may state or imply that blood or semen was identified on an item of evidence when a positive result is obtained from the appropriate confirmatory testing procedure(s).

4.2 Indication of Blood or Semen

The Examiner may state or imply that blood or semen was indicated on an item of evidence when a positive result is obtained from the appropriate presumptive testing procedure(s).

4.3 Inconclusive Result

An Examiner may state or imply that no determination can be made regarding the presence or absence of blood or semen when an inconclusive result is obtained from the appropriate testing procedure(s).

4.4 Negative Result

An Examiner may state or imply that no blood or semen was detected on an item of evidence when a negative result is obtained from the appropriate testing procedure(s).

4.5 Error Rate

An Examiner may state or imply that the analytical processes and procedures used to support serology testing do not have a calculable error rate due to the unpredictability of human error. An Examiner may further explain that the FBI Laboratory has a quality system in place to minimize and/or identify potential procedural errors.

4.6 Limitations

4.6.1 Confirmatory Tests

An Examiner may state or imply that confirmatory testing procedures may yield false-negative results (i.e., no test signal when blood or semen is present) due to the sensitivity of such tests.

4.6.2 Presumptive Tests

An Examiner may state or imply that presumptive testing procedures may yield false-positive results (i.e., test signal in the presence of materials other than blood or semen) due to the lower specificity of such tests.

4.6.3 Negative Results

An Examiner may state or imply that the recovery of an insufficient quantity of blood or semen for detection and/or the recovery of biological material of insufficient quality can limit the ability to detect blood or semen using both presumptive and confirmatory testing procedures.

5 Statements Not Approved for FBI Serological Examination Testimony and/or Laboratory Reports

5.1 Numerical Certainty

An Examiner may not state or imply that a level of numerical certainty is calculated to support the presumptive identification of blood or semen.

5.2 Zero Error Rate

An Examiner may not state or imply that serological examinations have a zero error rate or are infallible.

5.3 Reasonable Certainty

An examiner shall not use the expressions ‘reasonable degree of scientific certainty,’ ‘reasonable scientific certainty,’ or similar assertions of reasonable certainty in either reports or testimony unless required to do so by a judge or applicable law.

6 Laboratory Report Reviews

The content of a *Laboratory Report* will be reviewed according to the appropriate LOM practices and the appropriate DNA procedures to ensure compliance with the standards contained within this document.

7 Testimony Reviews

Testimony provided by DNA personnel will be reviewed in accordance with the LOM *Practices for Testimony Related Activities* to ensure compliance with the standards contained within this document.

8 References

FBI Laboratory Operations Manual

DNA Procedures Manual

United States. Department of Justice. Office of Legal Policy. *Forensic Science. Department of Justice Uniform Language for Testimony and Reports for the Forensic Serological Examinations*. Retrieved from the Department of Justice Web site:
<https://www.justice.gov/olp/uniform-language-testimony-and-reports>.

Rev. #	Issue Date	History
1	02/28/18	<p>Changed title to remove DNA Casework Unit and change Identifications to Examinations.</p> <p>Updated to include BAU throughout.3.3 and 3.4: clarified the responsibilities of the individuals in accordance with the LOM.</p> <p>4: added paragraph explaining that tests are used in conjunction</p> <p>4.5: moved from 5.2 and clarified</p> <p>5.1: added “presumptive”</p> <p>5.3: moved from 4.5</p>
2	03/27/19	<p>Updated to comply with Department of Justice (DOJ) Uniform Language for Testimony and Reports (ULTR) for Forensic Serological Examinations.</p> <p>1, 2: updated language to clarify.</p> <p>3.4: changed “individual’s manager” to “authorized evaluator” to mirror LOM revisions.</p> <p>4: reworded to clarify.</p> <p>4.2: updated to “Indicated” to mirror the DOJ ULTR.</p> <p>5.3 removed Absolute Non-identification section, replaced with Reasonable Certainty section.</p> <p>7: updated LOM name of practice.</p> <p>8: added ULTR reference.</p>

Approval

Redacted - Signatures on File

DNA Technical Leader

Date: 03/25/2019

Acting BAU Chief

Date: 03/25/2019

DCU Chief

Date: 03/25/2019

QA Approval

Quality Manager

Date: 03/25/2019

FBI Approved Standards for Scientific Testimony and Report Language for Autosomal DNA Testing

1 Purpose

This document provides examples of statements that are approved for reporting scientifically supported conclusions and offering expert opinions during testimony by Forensic Examiners representing the FBI Laboratory's DNA Casework Unit (DCU) and Scientific and Biometrics Analysis Unit (SBAU) DNA Group. It is noted that these examples are not intended to be all inclusive and may be dependent upon the precedent set by the judge or locality in which a testimony is provided. Further, these examples are not intended to serve as precedent for other forensic laboratories and do not imply that statements by other forensic laboratories are incorrect, indefensible, or erroneous.

2 Scope

This document applies to Forensic Examiners who prepare an FBI *Laboratory Report* (7-1, 7-1 LIMS, 7-273, or 7-273 LIMS) and/or provide testimony related to autosomal DNA examinations.

3 Responsibilities

3.1 The Examiner will ensure that a *Laboratory Report* is consistent with the approved language contained within this document.

3.2 The Examiner will ensure that his/her testimony related to autosomal DNA examinations is consistent with the standards contained within this document.

3.3 The Technical Reviewer will ensure that a DNA *Laboratory Report* contains language consistent with the standards contained within this document.

3.4 An authorized evaluator will assess if testimony provided by DNA staff complies with the statements contained within this document in accordance with the FBI Laboratory Operations Manual (LOM) *Practices for Testimony Related Activities*.

4 Statements Approved for FBI Autosomal DNA Testimony and/or Laboratory Reports

4.1 Inclusion/Cannot Exclude/Match

An Examiner may state or imply that a known individual is included as a possible contributor to the DNA evidence when the DNA typing results obtained from the evidence sample and the known contributor are the same or when the DNA results from a known reference sample are

present in a mixture. Each DNA inclusion must be clearly and properly qualified with either a statistic or a qualitative statement.

When statistics are provided, STRmix™ analysis is performed to provide a likelihood ratio (LR). The lowest calculated LR from applicable population groups is reported. When this LR is > 1 , support for an inclusion is reported. A qualitative statement, which verbally describes the amount of support for the inclusion, based on the LR, may be provided.

<u>Likelihood Ratio:</u>	<u>Qualitative Equivalent:</u>
2 to <100	Limited support for Inclusion
100 to $<10,000$	Moderate support for Inclusion
10,000 to $<1,000,000$	Strong support for Inclusion
$\geq 1,000,000$	Very strong support for Inclusion

A qualitative statement of inclusion that is not supported by a statistical calculation must be limited to situations in which the presence of an individual's DNA on an item is reasonably expected. The provenance of the sample must be established in the case record when statistics are not calculated.

4.2 Inconclusive or Uninformative

An Examiner may state or imply that no conclusion can be provided for a comparison between the DNA typing results obtained from an evidentiary sample and a known reference sample. When the lowest calculated LR from applicable population groups is one, this conclusion is generally reported as uninformative.

4.3 Unsuitable Results

An Examiner may state or imply that the DNA typing results are not suitable for comparisons when:

- a sample amplified using the GlobalFiler™ (GF) kit results in a mixture of 5 or more individuals,
- a sample amplified using the Identifiler Plus® (ID+) kit results in a mixture of 5 individuals without an assumed contributor, or
- a sample amplified using the ID+ kit results in a mixture of more than 5 individuals.

4.4 Exclusion / Support for Exclusion

An Examiner may state or imply that a known individual is excluded as a possible contributor to the DNA obtained from the evidence when the DNA profile obtained from the evidence and the known reference sample are different (i.e., a visual exclusion).

An Examiner may state or imply that a known individual is excluded as a possible contributor to the DNA obtained from the evidence when the LR calculated with STRmix™ results in an $LR \leq 1/100$ (i.e., 0.01). The calculated LR will not be included in the report, but it will be maintained

in the casefile and may be stated during testimony.

An Examiner may state or imply that there is support for an exclusion when the LR calculated with STRmix™ results in the following: $1/100 < LR \leq 1/2$ (i.e., $0.01 < LR \leq 0.5$). In this instance, the LR is included in the report. A qualitative statement, which verbally describes the amount of support for the exclusion, based on the LR, may be provided. There is limited support for the exclusion when $1/100 < LR \leq 1/2$.

4.5 Mixtures

An Examiner may state or imply that a mixture of DNA was obtained from an evidentiary sample. The assumed number of contributors to a mixture may be provided in the report and stated during testimony.

4.6 Sex Determination

An Examiner may state or imply that the DNA typing results indicate the presence of female DNA, male DNA, a mixture of male and female DNA, or that no conclusion regarding sex typing results can be provided.

4.7 Familial Comparisons

An Examiner may state or imply biological relatedness based on the probability of the DNA profile results when compared under two mutually exclusive hypotheses. Statements of biological relatedness will be supported by a statistical estimate [likelihood ratio (LR) or combined kinship index (KI)] with a value > 100 generally supporting the hypothesis of relatedness.

When the KI is between 1 and 100, the Examiner may state or imply that there is insufficient support to conclude relatedness.

Statistical estimates with values < 1 generally support the hypothesis of unrelatedness; however, the conclusion drawn is dependent on the relatives provided:

- The most informative pedigrees include at least one parent or one child, or at least two full siblings. When the KI is < 1 for these pedigrees, an Examiner may state or imply that biological relatedness is unlikely.
- Less informative pedigrees include only one full or half sibling, or more distant relatives. When the KI < 1 for these pedigrees, an Examiner may state or imply that there is insufficient support to conclude relatedness.

4.8 Other Nuclear DNA Conclusions

An Examiner may state or imply that no DNA typing results were obtained from the evidence or that no DNA typing results foreign to or unlike an individual whose DNA is reasonably expected to be present were obtained from the evidence.

An Examiner may state or imply that the DNA typing results obtained from the evidence are suitable for comparison purposes when no reference samples are available for comparison.

4.9 False Inclusions

An examiner may state or imply that the probability of observing an LR of x or larger from an unrelated non-donor is no more than 1 in x .

5 Statements Not Approved for FBI Autosomal DNA Testimony and/or Laboratory Reports

5.1 Absolute Identification

An Examiner may not state or imply that a match provides an absolute identification of the individual from whom the biological material originated.

5.2 Reasonable Degree of Scientific Certainty

An Examiner may not state or imply that any conclusion is “to a reasonable degree of scientific certainty” unless required by a judge or applicable law.

5.3 Racial/Ethnicity Prediction

An Examiner may not state or imply that an autosomal DNA profile can be used to predict the specific population, racial, or ethnic group to which an individual belongs.

5.4 Zero Error Rate

An Examiner may not state or imply that forensic autosomal DNA examinations have a zero error rate or are infallible.

6 Laboratory Report Reviews

The content of a *Laboratory Report* will be reviewed per the appropriate LOM practices and the appropriate DNA Unit Standard Operating Procedures (SOPs) to ensure compliance with the standards contained within this document.

7 Testimony Reviews

Testimony provided by DNA staff will be reviewed in accordance with the LOM *Practices for Testimony Related Activities* to ensure compliance with the standards contained within this document.

8 References

FBI Laboratory Operations Manual

FBI Laboratory *DNA Procedures Manual*

Office of the Attorney General Memorandum, Recommendations of the National Commission on Forensic Science, September 6, 2016

Bright, J-A, et.al. Internal validation of STRmix™ – A multi laboratory response to PCAST. *Forensic Science International: Genetics* 24 (2018) 11-24.

SWGDAM. Recommendations of the SWGDAM Ad Hoc working group on genotyping results reported as likelihood ratios. 2018. Available at www.swgdam.org.

Rev. #	Issue Date	History
2	09/24/18	Updated to reflect SWGDAM recommended verbal scale for both ID+ and GF amplification kit procedures. Changes include: 1 and 2: clarified 3.3: changed from Administrative to Technical reviewer 4.1: added verbal scale for LR > 1 4.2: defined LR = 1 as uninformative 4.4: defined LR ≤ 1/100 as an exclusion, and 1/100 < LR < 1/2 as limited support for an exclusion 4.5: removed source attribution section and renumbered 4.6: simplified language 4.9: removed Error Rate section and added False Inclusions section 5.1: removed reference to source attribution Added two references
3	10/16/19	1: updated BAU to SBAU 3.4: changed manager to evaluator per LOM 3.4 and 7: updated LOM title to <i>Practices for Testimony Related Activities</i> 4.4: corrected 0.001 to 0.01 in one instance 5.4: updated language to be consistent with SOP 711 and ULTR

Approval

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DNA Technical Leader

Date: 10/09/2019

SBAU Chief

Date: 10/09/2019

DCU Chief

Date: 10/09/2019

QA Approval

Quality Manager

Date: 10/09/2019

FBI Approved Standards for Scientific Testimony and Report Language for Haplotype Testing

1 Purpose

This document provides examples of statements that are approved for reporting scientifically supported conclusions and offering expert opinions during testimony by Forensic Examiners within the FBI Laboratory's DNA Casework Unit (DCU) and Biometrics Analysis Unit (BAU) DNA Group. It is noted that these examples are not intended to be all inclusive and may be dependent upon the precedent set by the judge or locality in which a testimony is provided. Further, these examples are not intended to serve as precedent for other forensic laboratories and do not imply that statements by other forensic laboratories are incorrect, indefensible, or erroneous.

2 Scope

This document applies to Forensic Examiners who prepare an FBI *Laboratory Report* (7-1, 7-1 LIMS, 7-273, or 7-273 LIMS) and/or provide testimony related to haplotype DNA examinations.

3 Responsibilities

3.1 The Examiner will ensure that a *Laboratory Report* is consistent with the approved language contained within this document.

3.2 The Examiner will ensure that his/her testimony related to haplotype DNA examinations is consistent with the standards contained within this document.

3.3 The Technical Reviewer will ensure that a *Laboratory Report* contains language consistent with the standards contained within this document.

3.4 An authorized evaluator will assess if testimony provided by DNA personnel complies with the statements contained within this document in accordance with the FBI Laboratory Operations Manual (LOM) *Practices for Testimony Related Activities*.

4 Statements Approved for FBI Haplotype Testimony and/or Laboratory Reports

4.1 Inclusion/Cannot Exclude/Match

The Examiner may state or imply that an inclusion is the determination that two haplotypes, generally one from an evidence sample and one from a reference sample, may have originated from the same source or lineage because the haplotypes are concordant. Within a *Laboratory*

Report and during the course of testimony, an Examiner will further state or imply that an inclusion is not an identification because the lineage will share the haplotype and unrelated individuals may also share the haplotype. An inclusion will be supported by a statistical estimate (e.g., upper bound frequency estimate or likelihood ratio) and/or a qualitative statement.

A qualitative statement of inclusion which is not supported by a statistical calculation must be limited to situations in which the presence of an individual's DNA on an item is reasonably expected. The provenance of the sample must be established in the case record when statistics are not calculated.

4.1.1 Level of Certainty

An Examiner may state or imply a level of certainty in his/her calculation of the supporting statistic. The level of certainty is based on a 95% confidence interval.

4.2 Inconclusive

An Examiner may state or imply that no conclusion can be provided for a sample or for a comparison between haplotypes, generally those obtained from an evidentiary sample and a known reference sample. Such a conclusion is termed an inconclusive result and may be the consequence of an insufficient number of differences detected between two haplotypes.

4.3 Unsuitable Results

An Examiner may state or imply that the DNA typing results are not suitable for matching purposes when a Y-STR mixture cannot be attributed to individual contributors (i.e., the mixture is indistinguishable).

An Examiner may state or imply that the DNA typing results are not suitable for comparisons when a mitochondrial DNA profile indicates a mixture of two or more individuals.

4.4 Exclusion

An Examiner may state or imply that two haplotypes, generally one from an evidence sample and one from a reference sample, are excluded as originating from the same source or lineage when there are sufficient differences detected between the haplotypes.

4.5 Mixtures

An Examiner may state or imply that a mixture of DNA was obtained from an evidentiary sample. The minimum number of contributors to a Y-STR mixture may be provided in the report and stated during testimony.

4.6 Other Haplotype Conclusions

An Examiner may state or imply that no DNA typing results were obtained from the evidence or

that no DNA typing results foreign to or unlike an individual whose DNA is reasonably expected to be present were obtained from the evidence.

An Examiner may state or imply that the DNA typing results obtained from the evidence are suitable for comparison purposes if no reference samples are available for comparison.

4.7 Error Rate

An Examiner may state or imply that the analytical processes and procedures used to support DNA typing technology do not have a calculable error rate due to the unpredictability of human error. An Examiner may further explain that the Laboratory has a quality system in place to minimize and/or identify potential procedural errors.

5 Statements Not Approved For FBI Haplotype Testimony and/or Laboratory Reports

5.1 Absolute Identification

An Examiner may not state or imply that two matching haplotypes provide an absolute identification of the individual from whom the biological material originated.

5.2 Racial/Ethnicity Prediction

An Examiner may not state or imply that haplotype results can be used to predict the specific population, racial, or ethnic group to which an individual belongs.

5.3 Zero Error Rate

An Examiner may not state or imply that forensic haplotype-based DNA examinations have a zero error rate or are infallible.

5.4 Reasonable Certainty

An examiner shall not use the expressions ‘reasonable degree of scientific certainty,’ ‘reasonable scientific certainty,’ or similar assertions of reasonable certainty in either reports or testimony unless required to do so by a judge or applicable law.

6 Laboratory Report Reviews

The content of a *Laboratory Report* will be reviewed per the appropriate LOM practices and the appropriate DNA procedures to ensure compliance with the standards contained within this document.

7 Testimony Reviews

Testimony provided by DNA staff will be reviewed in accordance with the LOM *FBI Practices for Testimony Related Activities* to ensure compliance with the standards contained within this document.

8 References

FBI Laboratory Operations Manual

DNA Procedures Manual

United States. Department of Justice. Office of Legal Policy. Forensic Science. *Department of Justice Uniform Language for Testimony and Reports for the Forensic Mitochondrial DNA Examinations*. Retrieved from the Department of Justice Web site:
<https://www.justice.gov/olp/uniform-language-testimony-and-reports>.

United States. Department of Justice. Office of Legal Policy. Forensic Science. *Department of Justice Uniform Language for Testimony and Reports for the Forensic Y-STR DNA Examinations*. Retrieved from the Department of Justice Web site:
<https://www.justice.gov/olp/uniform-language-testimony-and-reports>.

Rev. #	Issue Date	History
1	02/28/18	<p>Updated Scope</p> <p>3.3 Changed Unit Chief or designee to Technical Reviewer.</p> <p>3.4 Changed Unit Chief or designee to the testifying individual's manager and Subject Matter Expert, when appropriate.</p> <p>4.1 Clarified when quantitative statements are not provided.</p> <p>4.2 Updated to reflect current mitochondrial DNA interpretation procedures.</p> <p>4.3 and 4.5 Added Unsuitable Results section and Mixtures section, renumbered remaining sections.</p> <p>4.4 Rephrased.</p> <p>4.6 Changed wording for conditioned specimen for consistency with Autosomal ASSTR.</p> <p>4.7 Added section rephrased from second sentence of 5.3.</p> <p>5.3 Removed second sentence.</p>
2	03/27/19	<p>Updated to comply with Department of Justice (DOJ) Uniform Language for Testimony and Reports (ULTR) for Forensic Mitochondrial DNA and Y-STR DNA Examinations.</p> <p>1: Clarified language.</p> <p>3.4: Changed "individual's manager" to "authorized evaluator" to mirror LOM changes.</p> <p>4.1: Added clarification regarding when additional explanation is required.</p> <p>4.2: Generalized language to encompass all haplotype comparisons.</p> <p>4.4: Changed sentence structure to mirror 4.2.</p> <p>5.3: Changed to include examinations as a whole.</p> <p>5.4: Included section on reasonable certainty.</p>

Approval

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DNA Technical Leader

Date: 03/25/2019

Acting BAU Chief

Date: 03/25/2019

DCU Chief

Date: 03/25/2019

QA Approval

Quality Manager

Date: 03/25/2019